THE RESISTANT MEMBRANES OF KERATIN FIBRES

by

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I certify that this thesis is the original work of the candidate, except where due credit is given to the work of others.

J. D. Reeder.

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SUMMARY

When wool fibres are immersed in aqueous chlorine solutions (the Allworden reaction), a thin surface membrane (the epicuticle) is raised in the form of hemispherical sacs.

It is shown that epicuticle consists of discrete membranes covering individually each cuticle cell, in contrast to the almost-universal belief that it forms a continuous sheath over the entire fibre. The discontinuous nature of epicuticle is shown to apply to a variety of keratin fibres of different surface structures. Formation of Allworden sacs on isolated cuticle material is used to differentiate between intact cuticle cells and cuticle fragments; this allows conclusions to be drawn regarding the arrangement of cuticle cells in the fibre surface.

The chemical resistance of epicuticle, particularly towards surface-degradative shrinkproofing reagents, is demonstrated by Allworden sac formation on pretreated wools. It is concluded that sac formation occurs because of concurrent oxidation of cystine and hydrolysis of peptide bonds by the chlorine, resulting in formation of soluble protein degradation products under the semi-permeable membrane.

Aqueous bromine solutions raise a thicker membrane

than chlorine; the results of some comparative studies using this reagent are presented.

Resistant membranes have been isolated from Merino wool by selectively dissolving the rest of the fibre. Because these whole-fibre membranes and epicuticle have similar chemical and physical properties, it is postulated that epicuticle is the outer resistant fraction of the unit cell membrane which surrounds the cuticle cells. The Allworden reaction is shown to be very dependent on the quantity of oxidisable cystine present; this explains why sacs form only on the (sulphur-rich) exocuticle side of cuticle cells and not at all on cortical cells.

An estimate is made of the amount of 'cell membrane complex' present in keratin fibres; the importance of this complex in diffusion phenomena is demonstrated experimentally. These results indicate that epicuticle does not necessarily form the surface barrier to diffusion which was originally identified with the 'continuous epicuticular sheath' concept. Emphasis is given to the need for re-evaluation of the mechanisms underlying many surface phenomena, particularly in terms of the discontinuous nature of epicuticle.

Preliminary results are presented for the separation and analysis of (non-keratinous) endocuticle, and the possibilities of separating fibres into keratin and non-keratin fractions are discussed.

CONTENTS

1. THE HISTOPHYSICAL AND HISTOCHEMICAL STRUCTURE OF WOOL

[A] WOOL KERATIN:

Wool is identified with other fibrous proteins such as horn, hooves, hair and feathers, as a member of the 'keratin' family. Keratins normally occur as consolidated dead cellular residues which are tough, elastic and insoluble in dilute aqueous media. Their general resistance to proteolytic enzymes led Block and Vickery (1930) to suggest this resistance as a criterion for distinguishing keratin from other proteins. Solvent-cleaned keratin consists of approximately 98% protein plus small amounts of lipids and mineral matter.

Although all keratins have similar amino acid compositions, wool keratins from different sources show slight differences in chemical composition and histological structure. Most of the work described in this thesis was carried out on Merino 64's wool i.e. fine quality wool of approximately 20μ diameter ($10^{4}\text{Å} = 1\mu = 10^{-4}$cm); keratin fibres from other sources were used only where special

structural features were pertinent to the investigation. The next few pages are devoted to a brief summary of the relevent physical and chemical composition of fine wool fibres - the general structural features are applicable to other keratin fibres.

[B] HISTOLOGICAL STRUCTURE:

Figure 1.1, page 2a, is a scanning electron micrograph illustrating the exterior of a typical Merino wool fibre. The internal structure is extremely complex, and a large number of recognisable levels of organisation have been identified. The two major components are the laminated outer protective covering (the cuticle) and the main central portion of the fibre (the cortex). Figure 1.2, page 2b, shows schematically the main features of the cuticle and cortex.

(i) Cuticle:

The cuticle consists of flattened overlapping cells arranged similarly to tiles on a roof, with the overlapping edges of these cells or 'scales' pointing towards the tip of the fibre. For a fibre of 20μ diameter, the scales have the following approximate dimensions - width 30-50μ, visible length 10-20μ and thickness 0.5-1.0μ. It will be shown in chapter 2 that the relative size and arrangement of these cells varies with different keratin fibres.

Figure 1.1. Merino Wool Fibre - Scanning Electron Micrograph.

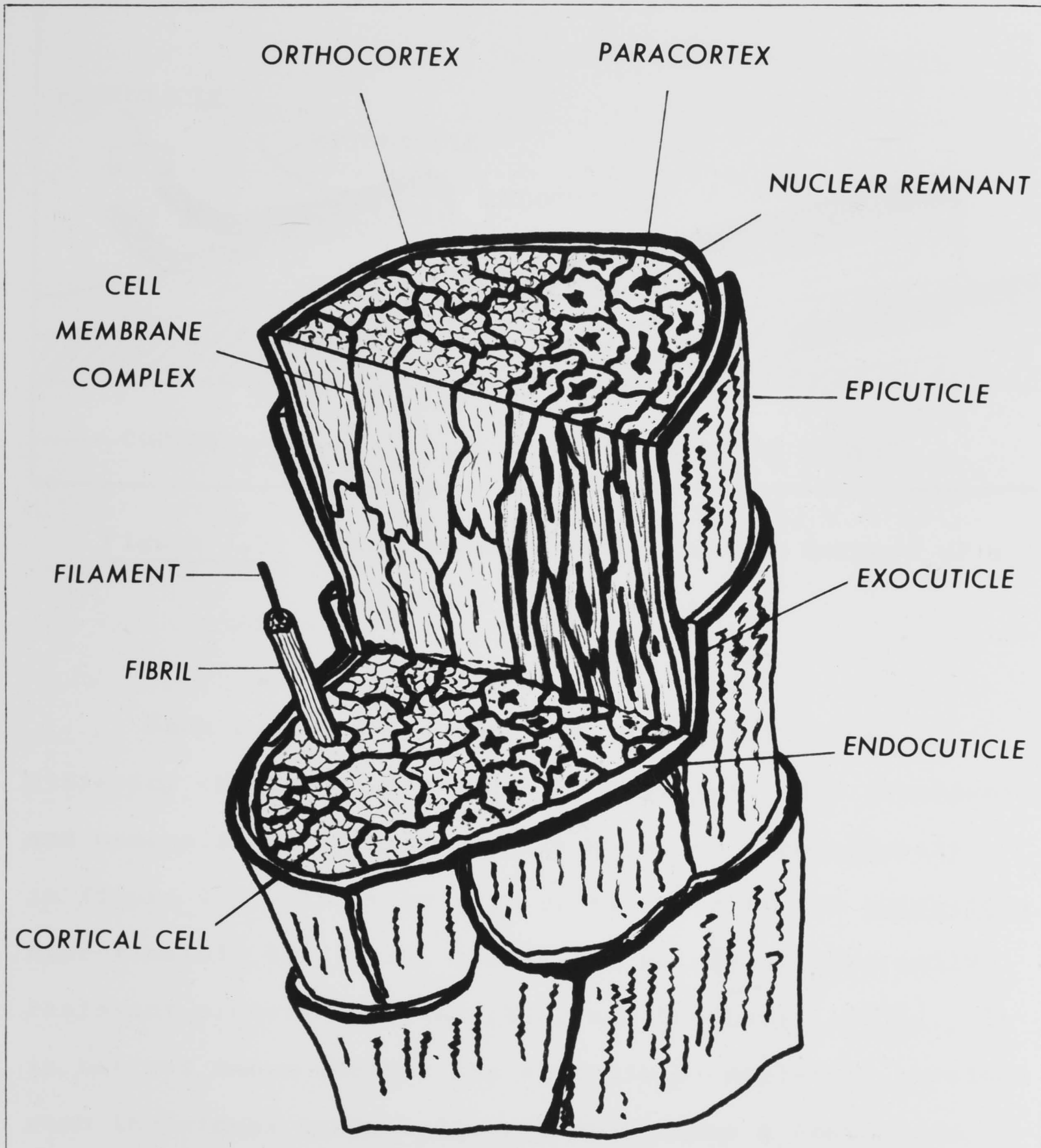

Figure 1.2. Histological Components of Merino Wool (from Dobb et al. (1961))

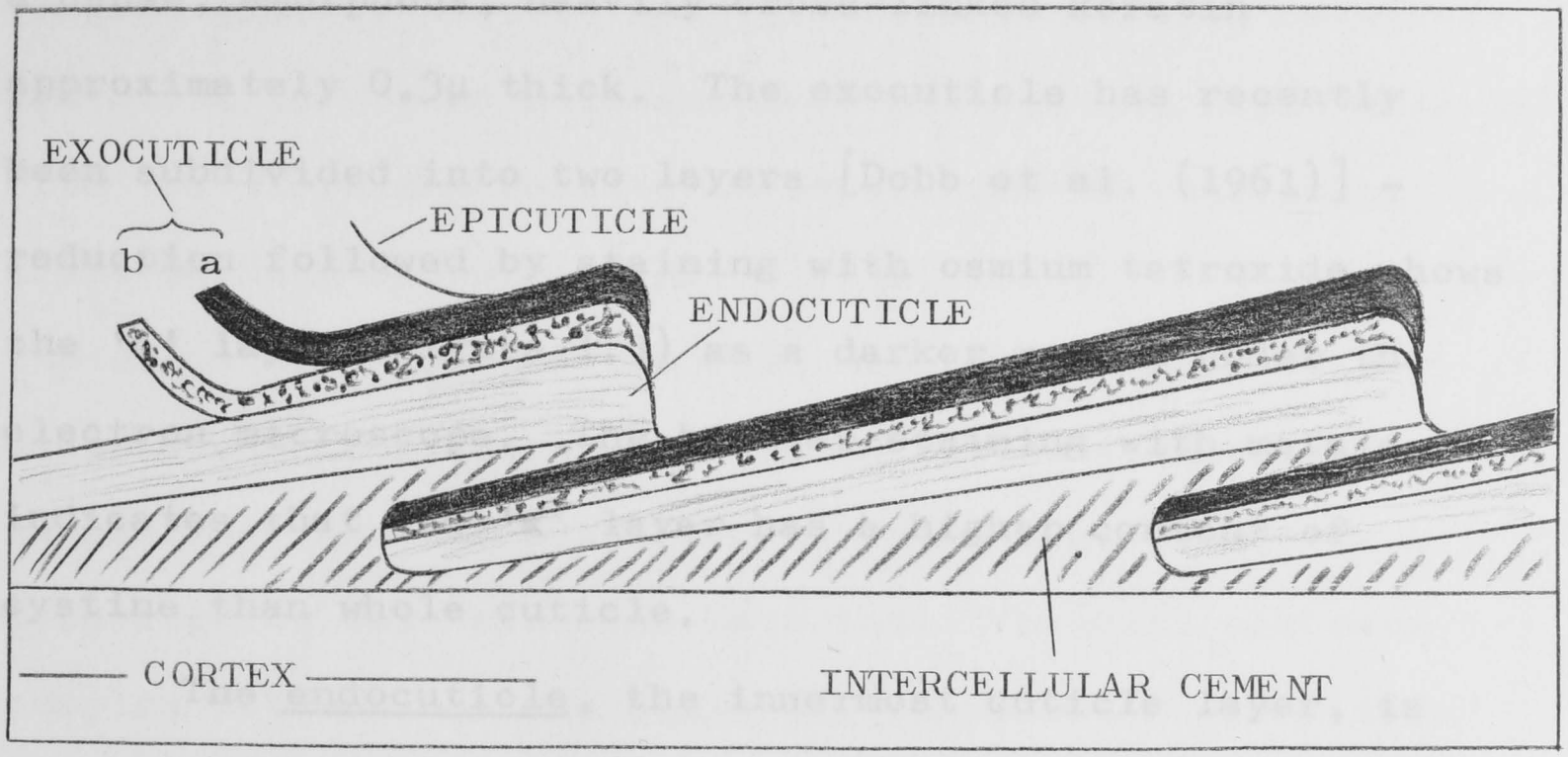

Figure 1.3. Longitudinal Section of the Surface of a Wool Fibre (Schematic).

Each cuticle cell consists of several layers of differing chemical and physical properties. The arrangement and nomenclature of these layers are shown schematically in figure 1.3. The very thin outer layer is the epicuticle, approximately 40Å thick, consisting mainly of chemically-resistant protein material [King and Bradbury (1967)]. It is not yet known whether the epicuticle completely envelops each individual scale cell or simply forms a continuous outer sheath around the whole fibre. Much of this thesis is devoted to a comprehensive analysis of the chemical and physical properties of epicuticle.

Immediately beneath the epicuticle lies the exocuticle,

a dense, amorphous, heavily cross-linked keratin approximately 0.3μ thick. The exocuticle has recently been subdivided into two layers [Dobb et al. (1961)] - reduction followed by staining with osmium tetroxide shows the 'a' layer (figure 1.3) as a darker region under the electron microscope. The heavier staining with metals indicates that the 'a' layer has a higher content of cystine than whole cuticle.

The endocuticle, the innermost cuticle layer, is approximately 0.3μ thick and consists of 'modified cellular residue' [Mercer (1961)] of low sulphur content and correspondingly reduced cross-link density. Its resistance to keratinolytic reagents supplements that of the exocuticle and so increases the total protective effect of the cuticle against chemical action. The dissolution of endocuticle by enzymes is the basis of attempts to separate and analyse exo- and endocuticle (see Appendix).

The prefixes epi-, exo- and endo- were suggested by Lindberg et al. (1949), following the nomenclature used to describe equivalent subdivisions of the insect cuticle.

The protein and lipid layers of the original cell membranes, together with a layer of intercellular cement, form the boundaries between cuticle cells, cortical cells, and between cuticle and cortex. These layers form what is sometimes referred to as the 'cell membrane complex'

[Rogers (1964); Mercer (1965)]. Ultrasonication or severe agitation in formic acid extracts protein and lipid material from the fibre and results in separation of individual scale cells [Bradbury et al. (1965a, 1966, 1967)]. This indicates that part of the cell membrane complex is dissolved or considerably modified by the formic acid, suggesting that the scales occur as discrete entities separated by cell membrane material. The cell membrane complex between cuticle cells may therefore be considered as part of the wool fibre cuticle. The protein:lipid ratio of the extract depends on the severity of the formic acid extraction [Bradbury et al. (1965a, 1967)] - this may account for variations between 1% and 8% (of the weight of wool) in reported estimations of cell membrane material. Clearly, the terms 'cell membrane', 'cell membrane complex' and 'intercellular cement' require closer definition and characterisation - different workers have differing views on the origin and composition of the intercellular material in keratin fibres. These aspects are considered in greater detail in chapter 3.

(ii) Cortex:

The cortex, representing approximately 90% of the wool fibre, is composed of spindle-shaped cell residues 80-100μ long and 3-6μ in diameter. These cortical cells are surrounded by the cell membrane complex referred to

above. Most of the mechanical and physical properties of wool fibres are conferred by the cortical cells.

Each cortical cell consists of several fibrils, which in turn are made up of filaments of approximately 80Å diameter, surrounded by amorphous protein containing a high proportion of sulphur - the matrix. Rogers and Filshie (1963) have shown by electron microscopy, using suitable staining techniques, that the filaments consist of still smaller sub-units (protofilaments), each of the order of 20Å in diameter. These are probably arranged as a circle of 9 protofilaments surrounding 2 central protofilaments (termed the '9+2' structure), again embedded in an amorphous sulphur-rich matrix.

The X-ray and infra-red studies of Fraser et al. (1964, 1965) indicate that a modified 3-strand rope structure (i.e. three α-helical polypeptide chains in the form of a coiled-coil), as originally suggested by Crick (1952, 1953), is the probable structural organisation of the protofilaments.

In accordance with the recommendations of Mercer et al. (1963), the terms 'fibril', 'filament' and 'protofilament' are used here in place of the alternative terms 'macrofibril', 'microfibril' and 'protofibril', respectively.

Nuclear remnants are deposited near the centre of

each cortical cell as a result of degeneration of the original cell nucleus during keratinisation.

This completes a brief summary of the known histological features of fine Merino wool. Coarser wool fibres and many other hair and fur fibres contain, in addition to cuticle and cortex, a third major component - the medulla. This occurs as a central core of amorphous protein of low sulphur content [Bradbury and O'Shea (1969)].

Table 1.1 lists average dimensions of some wool fibre components.

TABLE 1.1

REPRESENTATIVE WOOL FIBRE DIMENSIONS*

1,000,000	Å	-	length of cortical cell
200,000	"	-	diameter of wool fibre
40,000	"	-	diameter of cortical cell
5,000	"	-	diameter of fibril
250	"	-	thickness of cell membrane complex
80	"	-	diameter of filament
40	"	-	thickness of epicuticle
20	"	-	diameter of protofilament
10	"	-	diameter of α-helix
1.5	"	-	atomic spacing

* adapted, from Lundgren and Ward (1963).

In addition to the subdivisions listed above, the cortex of Merino wool fibres exhibits bilateral asymmetry. Thus Horio and Kondo (1953) found bilateral differences after staining with acid or basic dyes, and after swelling with alkali. Mercer (1953) proposed the names '<u>orthocortex</u>' and '<u>paracortex</u>' for these hemicylindrical segments (see figure 1.2); orthocortex refers to the more accessible, more reactive segment. The considerable amount of work done on ortho-para differentiation has been extensively reviewed by Chapman (1967).

Table 1.2 presents estimated proportions of the various histological components of Merino wool.

TABLE 1.2

WOOL FIBRE COMPONENTS*

Component	Weight %
Epicuticle	0.1
Exocuticle (a-layer	2
Exocuticle (b-layer	3
Endocuticle	5
Cell membrane complex	4
Microfibrils	55
Matrix	25
Nuclear remnants and other cell debris	6

* Averaged from best data available, in particular from Bradbury and King (1967), and Lundgren and Ward (1963).

[C] CHEMICAL COMPOSITION:

Unmodified wool keratin is insoluble in normal protein solvents, mainly because of the high content of disulphide cross-links. Oxidative or reductive fission of these cross-links followed by treatment with solvents which break hydrogen bonds results in extraction of soluble proteins, which can be fractionated into several components [see, e.g., Crewther et al. (1965)]. These protein fractions fall into two main classes, having a higher, and a lower, content of cystine sulphur than the whole fibre. There is some evidence to suggest that the low-sulphur proteins occur in the crystalline regions of the fibre (the filaments); and that the high-sulphur proteins are derived from the amorphous matrix material [Rogers (1964); Crewther et al. (1965)] but this has yet to be established unequivocally [Bradbury et al. (1967)].

The above approach has so far been applied only to the intact fibre, with the resultant difficulty of establishing exactly which region of the fibre supplied the extracted protein. A more realistic approach would seem to be to apply the above techniques after separation and isolation of the various histological components. This approach should eventually lead to complete characterisation of the various keratins by elucidation of the amino acid sequence of pure homogeneous protein fractions.

The major difficulty has been that chemical degradation during separation of the components, plus possible lack of purity of the preparations, has resulted in wide diversity in the many published amino acid analyses. The most recent and most successful attempts to isolate and analyse the various histological components are those of Bradbury and co-workers in this department - these workers have developed mild isolation procedures, resulting in minimal chemical degradation.

Table 1.3 summarises the results so far obtained. The methods used and detailed discussion of the conclusions drawn from these results have recently been reviewed [Bradbury et al. (1967), Chapman (1967), King (1967)]. Briefly, the main features are -

(i) epicuticle has a unique but unexceptional amino acid composition, leaving unexplained the reason for its extreme chemical inertness; (ii) whole cuticle is deficient in amino acids which participate in formation of α-helices, and has a preponderence of amino acids which prefer not to exist in α-helical segments, thus supporting other evidence that the cuticle is amorphous in nature; (iii) the inter-cellular cement has a low content of cystine - this explains why it is labile and easily extracted; (iv) because the cortex forms approximately 90% by weight of Merino fibres, it has a similar amino acid composition to that of whole wool.

TABLE 1.3

AMINO ACID ANALYSES OF MERINO WOOL COMPONENTS (MOLE %)

Amino Acid	Whole Wool[c]	Epi-cuticle[d]	Whole Cuticle[c]	Inter-Cellular Cement[e]	Cortical Cells[c]
Alanine	5.3	4.6	5.6	6.2	5.6
Arginine	6.8	4.3	4.8	6.2	6.8
Aspartic Acid	6.4	5.8	3.9	7.3	6.8
Citrulline[a]	0.1	0.9	0.5	0.4	0.1
Cysteic Acid	0.1	11.6[b]	0.4	0.1	0.2
Half-cystine	10.5	0.3[b]	14.4	2.1	9.2
Glutamic Acid	11.9	10.7	8.9	10.5	11.7
Glycine	8.6	15.4	9.6	14.4	9.5
Histidine	0.9	1.0	1.0	1.7	0.8
Isoleucine	3.1	2.5	2.5	3.9	3.3
Leucine	7.7	5.5	6.0	8.2	7.8
Lysine	3.1	4.8	2.9	4.6	2.9
Methionine	0.5	0.1	0.4	1.2	0.5
Phenylalanine	2.9	1.8	1.9	4.3	3.1
Proline	5.9	5.8	9.3	4.0	5.9
Serine	10.2	13.6	13.5	8.3	10.3
Threonine	6.5	3.6	4.9	4.9	5.6
Tyrosine	4.0	2.0	2.9	6.2	4.2
Valine	5.5	5.7	6.6	5.5	5.7

[a] Includes value for hydrolytic degradation product (ornithine).

[b] Method of isolation results in oxidation of cystine.

[c] Bradbury et al. (1965b).

[d] King and Bradbury (1967).

[e] Bradbury et al. (1965a).

Separation and analysis of ortho- and para-cortical cells have also been achieved [Chapman and Bradbury (1968)]. Surprisingly, only small differences in amino acid composition were found - these differences were considered to be insufficient to account for the considerable differences in the properties of the two cortical segments.

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2. STUDIES ON THE EPICUTICULAR MEMBRANE.

[A] HISTORICAL:

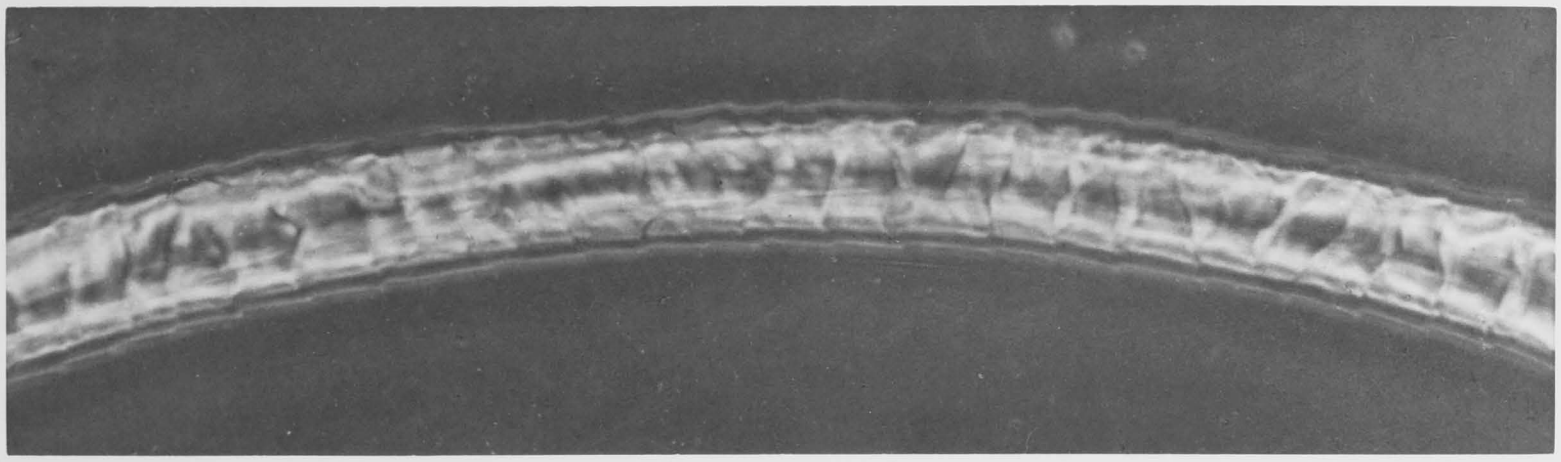
More than 80 years ago, McMurtrie's studies on fibre histology led him to suggest that the scales of undamaged wool fibres were held in place by a continuous surface membrane [McMurtrie (1886)]. Allworden (1916) found that treatment of virgin wool with aqueous chlorine solutions resulted in formation of characteristic bubbles or sacs on the fibre surface; the membrane enclosing these bubbles was called the 'epicuticular membrane' by Lindberg et al. (1949). The careful and comprehensive work of Muller (1939) established that these 'Allworden bubbles' are formed by a thin membrane situated on the surface of the scales. The sacs usually appear optically empty, but Brownian movement has been observed after staining with Methylene Blue, indicating the presence of a liquid under the membrane [Kronacher and Lodemann (1930)]. Thus the unstained bubbles are visible because of a difference in refractive index between the solutions inside and outside the membrane.

The enclosed liquid flows out if the sac is punctured by a microscope needle, leaving pieces of membrane still attached to the fibre [Kronacher and Saxinger (1925,1926)]. Bubble formation apparently arises from the semi-permeable nature of the membrane. Following Stirm and Colle(1935a,b,c), Muller (1939) postulated that chlorine reacts with protein in the scale layer immediately below the inert membrane, producing soluble macromolecular degradation products which then cause swelling under the membrane due to osmotic forces. Thus Hock et al. (1941a,b) found that addition of concentrated salt solution resulted in collapse of the bubbles. The electron microscopy of Ames (1952) indicates that the 'a' layer of the exocuticle is attacked by chlorine solutions, while Blackburn (1960) obtained peptides but no free amino acids from the contents of the Allworden sacs.

Hock et al. (1941a) commented that since the swellings caused by chlorine solutions are membranes filled with liquid, 'sac' is a more appropriate descriptive term than 'bubble', which is in widespread use in the literature.

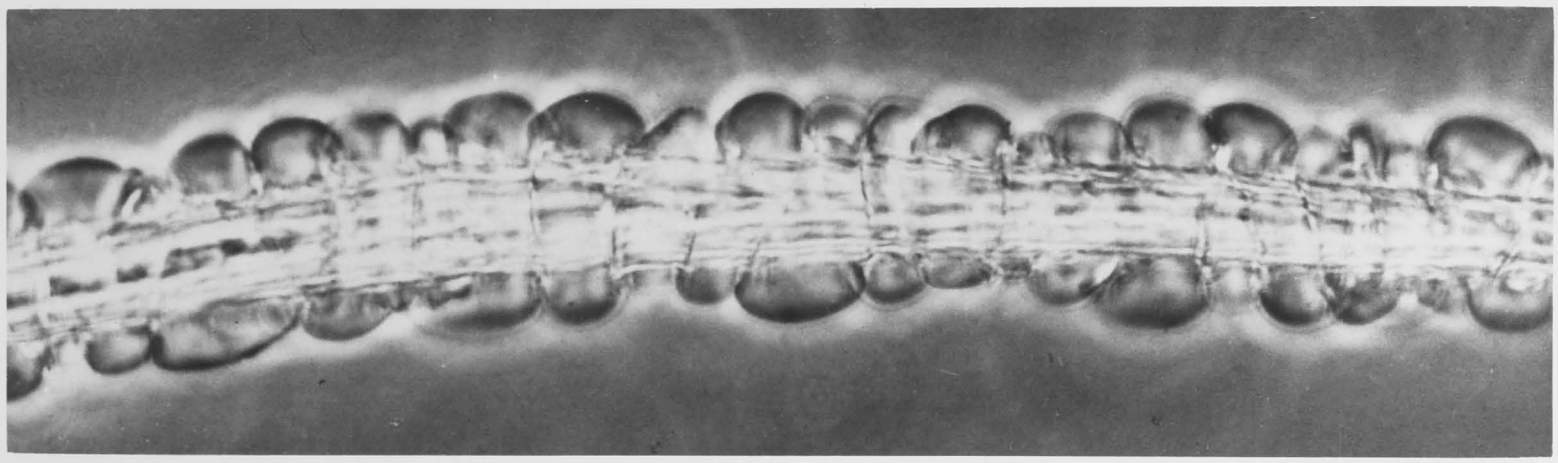
Aqueous bromine solutions also produce bubbles or sacs on the wool fibre surface [Herbig (1919)]. These sacs are superficially similar to those produced by the Allworden reaction.

Typical Allworden and Herbig sacs are shown in figures 2.2 and 2.3 respectively. Phase contrast was



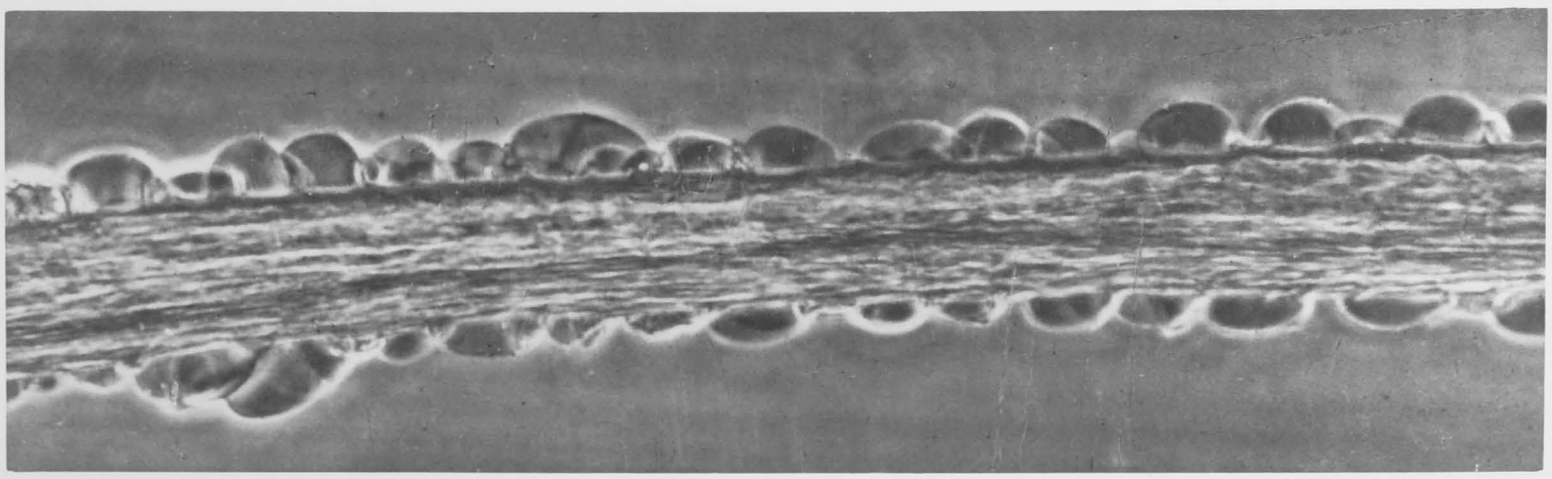
20μ

Figure 2.1. Merino Fibre Immersed in Water.



20μ

Figure 2.2. Merino Fibre Immersed in Chlorine Water.



20μ

Figure 2.3. Merino Fibre Immersed in Bromine Water.

employed to give greater optical contrast between the sac + contents and the surrounding liquid. For comparison, an untreated Merino fibre mounted in water is shown in figure 2.1.

A long-standing controversy exists over whether epicuticle covers the whole fibre as a continuous extra-cellular sheath, or is discontinuous in the sense that it covers or surrounds each individual cuticle cell. From the large number of opinions and arguments put forward, the various groups of researchers throughout the world 'vote' approximately 22 to 6 in favour of the continuous sheath concept. Thus Fraser and Rogers (1955a) believe the (continuous) epicuticle membrane is folded or tucked under each scale edge, probably cemented in place in some as yet undetermined way. In support of this, Dobb et al. (1961) state - "all available evidence suggests that a continuous sheath, formed by fusion of the epicuticle at the cell junctions, covers the external surfaces of the cuticle cells." The 'clothes-peg' function of the overlapping cuticle cells is a necessary postulate to explain the apparent association of the Allworden sacs with individual scales (see figure 2.2).

Related to the above problem is that of the origin of epicuticle. It either occurs as a definite component of the individual cuticle cells, or as a superficial coating on the main cuticle layers. The latter theory is most favoured at present because it is readily identifiable with

the continuous sheath concept - many people also believe that exocuticle forms a continuous layer.

The most likely biological source of epicuticle is from cell membrane material. Cell membranes originally envelop all cells before and during fibre formation and are still present around cortical cells and between cuticle cells in the fully keratinised fibre. The idea of association of epicuticle with single scales derives from the possibility that cell membranes may still be present on the outside of the cuticle cells, thus forming the epicuticle. This was first suggested by Muller (1939). Most proponents of the continuous sheath concept suggest that the original cell membranes are fused together under the scale edges to form the continuous external membrane [see, e.g., Mercer (1957); Rogers (1959)]. Before the growing fibre reaches the skin surface it is coated with a greasy secretion from the sebaceous gland and with an aqueous salt solution from the sweat gland, thereby providing other possible sources of deposition.

From electron microscope studies, Lindberg et al. (1948) reported the thickness of epicuticle to be 50-100Å. These workers isolated the epicuticle by shaking wool in chlorine water. Using similar techniques, King and Bradbury (1967) arrived at a value of 20-40Å.

Because epicuticle constitutes less than 0.1% of the

wool fibre, chemical and physical studies on the intact and the isolated membrane have been difficult and the results have very often been ambiguous. Thus the chemical composition varies with the method of isolation and the types of pretreatment applied to the fibre. Lagermalm and Gralen (1951) reported that sugars as well as amino acids were present in a wool extract hydrolysate containing 30% of epicuticle, suggesting the presence of carbohydrates. Alexander (1950) postulated that epicuticle was made up of lipids, possibly cross-linked to form a three-dimensional stable network. This idea was supported by Elliott (1950). Following the observation that epicuticle was resistant to digestion with sodium sulphide [Lindberg et al. (1948)], Elliott and Manogue (1952) concluded that it was not proteinaceous, but was a sterol complex of cholesteryl and other esters deposited by the sebaceous gland. However, Allworden sac formation has been observed below the skin surface at the point in the follicle where hardening or keratinisation starts (i.e. at the point where cystine linkages start to form from thiol groups) [Hock et al. (1941a); Mercer (1949); Schuringa et al. (1952a,c)]. This point is below the level of the sebaceous gland opening, so the above-mentioned observation refutes the conclusions of Elliott and Manogue, and also eliminates one of the possibilities for biological origin of epicuticle.

Most other workers agree that protein is the main constituent of epicuticle [Schuringa et al. (1952b); Zahn (1952); Golden et al. (1955)]. The most recent and most complete analysis [King and Bradbury (1967)] shows 80% protein, 5% lipid, and 5% ash, with 10% of material unaccounted for. Carbohydrate was tested for but could not be detected, in agreement with the observation of Ultee et al. (1953). Details of the amino acid composition were given in table 1.3.

Although now proven to be mostly proteinaceous, epicuticle exhibits remarkable chemical inertness; many workers have variously found that it is resistant to attack by alkalies, strong acids, reducing agents, oxidising agents and enzymes [see, e.g., Lindberg et al. (1949); Mercer (1953)].

Isolated fragments of epicuticle are not visible under the optical microscope because of their extreme thinness, but the membranes can be observed even at low magnification using phase contrast [Mariner (1951); Algera (1952)]. Light- and electron-microscopy usually show epicuticle as a structureless membrane, but striations and ridges are sometimes observed [Schuringa and Algera (1950); Mariner (1951); Lagermalm (1954)], particularly on bromine-treated preparations. It is possible that the striations are developed during or after the isolation procedure.

Membranes which appear similar to the epicuticle of keratin fibres under the microscope, have been isolated from the surfaces of other protein materials such as human skin and fingernails [Lagermalm et al. (1951)] and feathers [Philip et al. (1950,1951)], but the physical (and chemical?) structure of these substrates apparently precludes the development of Allworden sacs.

X-ray diffraction studies have yielded very little information - Lindberg et al. (1949) found β-keratin plus non-keratin reflexions from epicuticle isolated by shaking fibres in chlorine water, while Lagermalm and Gralen (1951) found no evidence for α- or β-keratin in the membrane raised by bromine water.

The rate and extent of sac formation were found to increase with increasing halogen concentration [Hock et al. (1941a,b); Leveau and Cebe (1953)] and with decrease in pH [Millson and Turl (1950)]. Pretreatment temperature [Daveloose et al. (1960)] and type of chemical pretreatment [Hock et al. (1941a,b); Meeuse et al. (1950); Leveau and Cebe (1953)] also affect sac formation. The rate of reaction is markedly changed by treatments which modify cystine linkages [Hock et al. (1941a,b)], suggesting that the reaction mechanism is concerned in some way with the disulphide bonds.

Most epicuticle studies have been done on wool fibres,

the finer grades of which possess a cuticle layer one cell thick. An interesting observation is that when coarser animal hairs (having a multi-cellular cuticle) are immersed in bromine water, the entire cuticle appears to lift. Sometimes very large sacs form, on top of which scale junctions and overlaps can be clearly seen [Leveau et al. (1952); Parisot and Leveau (1953)]. This was confirmed by Fraser and Rogers (1955b), who found a gradation from 'pure' epicuticle sacs to whole cuticle swellings with increasing coarseness of fibre.

Another peculiarity of the bromine reaction is that the sacs always form first on the cuticle layer covering the more-reactive orthocortex of fine wools. This suggests that the site of bromine attack may be the outer cortical cells [Leveau et al. (1953b); Fraser and Rogers (1955b)]. However, Kassenbeck (1958) reported a similar effect with chlorine water, and presented electron micrographs to support his hypothesis that the ortho-para concept may apply to cuticle cells as well as cortical cells.

The role of epicuticle as a barrier to diffusion has received considerable attention. It is generally agreed that this component forms a surface barrier to the entry of dye molecules [Whewell and Woods (1944); Lindberg et al. (1949); Millson and Turl (1950); Speakman (1950); Karrholm and Lindberg (1956)]. This follows, of course,

from the theory that epicuticle is a continuous outer sheath.

Lindberg (1950) found that treatments which he considered to perforate or disrupt epicuticle also increase the rate of absorption of acids, again implicating epicuticle as a barrier to diffusion. However, Lindberg's results also show that the rate increases further with increasing severity of pretreatment, suggesting that the whole cuticle, or even the whole fibre, may be involved. Thus Kopke and Nilssen (1960) have demonstrated the existence of a cuticular barrier to penetration of dyes.

Virgin wool fibres do not stain with the basic dye Methylene Blue [Whewell and Woods (1944)], but after a 'mild' mechanical treatment such as rubbing a fibre between the palms of the hands, it began to take up dye. Similarly, most fibres which had undergone normal industrial processing readily absorbed Methylene Blue, indicating damage to the epicuticle. Staining tests with Methylene Blue are often used to detect surface modification of wool fibres [see, e.g., Brown (1959)]. The above observations led Speakman (1950) to the conclusion that epicuticle is only important on undamaged fibres and that this membrane had very little effect on diffusion properties after mechanical processing - Speakman quoted the example of soap-soda scouring followed by carding. However, current industrial trends towards milder and fewer operations between fleece and final product

may necessitate a reassessment of the role of an epicuticular barrier in processed wools.

Degradative surface shrinkproofing treatments are reported to remove or disrupt epicuticle, despite its established chemical inertness [Stewart and Whewell (1960)]. Gralen (1950) proposed that the change in feel or handle of a wool fibre following chemical surface treatments may result from uneven removal of epicuticle along the fibre. Gralen also suggested that removal of epicuticle may be responsible for the increase in fibre friction which produces shrink-resistant effects. The argument against this is that mechanical processes apparently disrupt epicuticle, yet seldom produce marked changes in handle and certainly do not confer a measurable degree of shrink-resistance.

The summary given above indicates that it is often difficult to distinguish between the influence of epicuticle and whole cuticle; different results and interpretations may also be obtained depending on whether bulk properties or single-fibre properties are being assessed.

From the above survey it is apparent that much research effort was expended on epicuticle and the Allworden reaction during the decade 1948-1958. During the last 10 years very little new information has become available.

However, whether epicuticle is an entirely external membrane or a membrane enclosing individually each flattened cuticle cell, remains a question of prime importance. As mentioned earlier, most workers believe epicuticle to be continuous (and 'tucked in' at each scale edge) despite the lack of an adequate explanation for its biological origin and formation. A necessary consequence of this belief is that every chemical (and physical) treatment which removes more than approximately 0.1% by weight of the wool fibre, must first remove, disrupt or perforate the epicuticle, despite its established chemical inertness - the ratio of total surface area to area of the ends of a cylinder of length 10 cm and diameter 20μ is of the order of 10^4:1, so anisotropic 'end' effects will not be significant.

Mercer (1953) has critically reviewed the literature published prior to 1953, and has assembled evidence which very strongly favours continuity of epicuticle. Since that time, his conclusions have not been challenged, and the case appears to be closed. However, the concept of epicuticle as discrete cell membrane residues still seemed, to the present author, the more logical explanation, so the case has been reopened. The remainder of this chapter is devoted to presentation of new results and interpretations on (i) the physical structure of epicuticle, leading naturally to (ii) study of the variation of sac pattern

with scale structure and (iii) the effects of chemical and physical pretreatments; part (iv) deals with investigations into the incompletely-understood mechanism of Allworden sac formation.

[B] "EPI OR NOT EPI' - DEFINITION OF EPICUTICLE:

The membrane raised by chlorine water was specifically named epicuticle by Lindberg et al. (1949). Herbig (1919) found that bromine water also caused formation of sacs which were superficially similar to those induced by chlorine water. Thus, in the early 1950's, the name 'epicuticle' became synonymous with the membrane raised by chlorine or bromine, e.g. although Lindberg defined epicuticle as the chlorine membrane, he subsequently used bromine for 'epicuticle' studies [Lindberg (1953a)].

Leveau et al. (1952) showed that the bromine membrane appeared to vary in thickness from pure epicuticle for very fine wools to whole cuticle for coarse wools and hairs. Fraser and Rogers (1955b) reached a similar conclusion and suggested that, for fine wool fibres, this membrane consisted of epicuticle plus the 'a' layer of the exocuticle, while King and Bradbury (1967) have established that the bromine membrane is approximately 5 times thicker than the chlorine membrane. On the other hand, there seems to be no doubt that chlorine raises a well-defined membrane which is

independent of the nature or origin of the particular keratin fibre.

Unfortunately, the erroneous view that the chlorine and bromine membranes are identical is still held by many workers, and is found in most recent textbooks and review articles. Thus the Herbig bromine membrane is variously referred to as the bromine Allworden membrane; the Allworden membrane; the bromine epicuticle; or simply, the epicuticle. King and Bradbury (1967) have clearly stated that epicuticle should be regarded as the membrane raised by chlorine water. This is the definition of epicuticle used in the present work, although many experiments have been carried out using bromine water, in order to compare the properties of the two layers, and to permit comparisons with earlier work using this reagent.

[C] MATERIALS AND METHODS:

(i) Keratin Fibres:

(a) Merino 64's virgin fleece wool was the same as that used in earlier sorption studies by the author, and was from the same source as that used for the histological work of Bradbury and co-workers in this Department [see e.g., Chapman (1967); King (1967)]. The (weathered) tip third of each staple was cut off and wool grease removed by Soxhlet-extraction with petroleum ether (b.p.60-80^{o}C)

for 8 hours. The resulting wool was freed of dirt and suint salts by washing 6 times in distilled water at 50°C, followed by 2 x 24 hour washes in deionised distilled water.

(b) Lincoln 36's, processed to remove gross impurities and short fibres (i.e. in the form of wool 'top') was supplied by CSIRO Division of Textile Industry. This sample has been used for recent histological studies in this Department [King, 1967)] and in earlier work by Bradbury (1960).

(c) Human hair was pigmented red-brown hair from a 9-year old girl, supplied by Dr. J. H. Bradbury.

(d) Kangaroo fur, from the belly of a female red kangaroo, was obtained from CSIRO Division of Wildlife Research.

(e) Platypus fur and platypus guard hair, from the Fisheries and Wildlife Department, Melbourne, were taken from the belly region and manually separated into the fine fur fibres and the coarser guard hairs. A 'pure' sample of tail guard hairs, from the same source, was also studied.

(f) Seal fur, from the head and chest regions of a female Alaskan seal, was provided by Dr. J. Menkart, Harris Research Laboratories, Washington, U.S.A.

(g) Seal guard hair, of unknown origin, was the gift of the Returned Soldiers and Sailors Woollen Mill, Geelong, Victoria.

Samples (b) - (f) were used only for small-scale experiments and were cleaned of dirt, grease and skin debris by gentle agitation in 1% aqueous Gardinol BW detergent [Bradbury and Chapman (1964)], followed by rinses in heptane and absolute alcohol. The Lincoln and human hair fibres were cut into approximately 2 cm lengths before cleaning, but no attempt was made to remove weathered tips from this group of keratin fibres,

(ii) Chemicals:

All chemicals, with the exception of proprietary products, were A.R. grade. Solvents which were used in gravimetric experiments were distilled before use to ensure freedom from residues.

(iii) Chlorine Allworden Reaction [Allworden (1916)]:

A few fibres (or one drop of an aqueous cellular suspension) were placed between a microscope slide and a cover slip, then chlorine water was run under the cover slip with the aid of an eye-dropper. For reaction-rate studies, the time taken for the appearance of sacs was noted and the mean of at least 6 such measurements was obtained for each sample. Saturated solutions of chlorine were readily obtained by bubbling chlorine gas through deionised distilled water at $0^{o}C$ and allowing the resultant solid mass to warm to room temperature, usually $20^{o}C$. Freshly prepared (less than 3 hours old) chlorine solutions were used.

(iv) Bromine Reaction [Herbig (1919)]:

Saturated aqueous solutions of bromine were freshly prepared daily by alternatively cooling and shaking a mixture of bromine and deionised distilled water. Sacs were produced on fibres or other preparations in a similar manner to that described above for chlorine water.

(v) Light Microscopy:

Sac formation was observed under phase contrast using a Leitz Dialux microscope. Photomicrographs were taken on 9 cm x 12 cm glass plates or sheet film using the Leitz plate camera attachment. Occasionally photomicrographs were taken using a Leitz Polaroid assembly which gave 9 cm x 12 cm prints.

(vi) Scanning Electron Microscopy:

Scanning electron microscopy was carried out either by Cambridge Instrument Company, Cambridge, England, or by technicians at the Defence Standards Laboratory, Melbourne. using a 'Stereoscan' microscope. This instrument uses television techniques to develop 20 times the resolving power of the optical microscope with a depth of field 300 times as great. It is therefore ideal for studying the uneven surface structure of keratin fibres. Samples for scanning electron microscopy were prepared by vacuum-coating with gold-palladium alloy.

(vii) Preparation of Fibre Cross-sections for Microscopy:

Bundles of fibres were mounted in a Hardy microtome and sections 10-20μ in thickness were cut with a sharp razor blade. Embedding materials were not used; the sections were simply 'dusted' onto a microscope slide.

(viii) Shrinkproofing Tests:

0.25 gm samples of loose wool were immersed in 50 cc of 0.1% aqueous Lux (low-titre commercial soap) or 0.1% Lux + 0.1% sodium carbonate [see, e.g., Anderson and Leeder (1966)], and shaken at room temperature on a laboratory shaker for the required time.

[D] PREPARATION OF WOOL SAMPLES:

(i) Untreated wool:

Wool designated 'untreated' is Merino 64's virgin fleece wool, ether extracted and water washed, i.e. the wool sample referred to under Section [C](i).

(ii) Potassium bromate/salt treatment [McPhee (1960b)]:

Air-dry wool was given a chlorination shrinkproofing treatment using 2% potassium bromate (on weight of wool) in saturated aqueous potassium chloride made 0.1N with respect to sulphuric acid. After reacting for 1 hour at 20°C and 25:1 liquor:wool ratio, the wool was rinsed, treated with 0.1% sodium bisulphite (antichlor) for 10 minutes at 20°C, then neutralised with dilute sodium bicarbonate solution. Weight loss was 0.7%.

(iii) Potassium permanganate/salt treatment [McPhee (1960b)]:

Air-dry wool was treated with 5% potassium permanganate (on weight of wool) in saturated aqueous potassium chloride at 20°C and 25:1 liquor:wool ratio until the permanganate colour had faded. After rinsing and clearing the residual manganese dioxide with an aqueous solution containing 10% acetic acid - 5% sodium bisulphite (on weight of wool) the sample was soaked overnight in 0.001N hydrochloric acid. Weight loss was 1.4%.

(iv) Sulphuryl chloride treatments [e.g. Farnworth (1955)]

(a) Air-dry wool was treated for 1 hour under reflux (200:1 liquor:wool ratio) with carbon tetrachloride containing 10% (v/v) sulphuryl chloride, then washed 4 times in carbon tetrachloride and 4 times in ethanol.

(b) Vacuum-dry wool was sealed in an evacuated glass tube containing 50% (v/v) dry sulphuryl chloride in dry carbon tetrachloride (200:1 liquor:wool ratio) then heated at 40°C for 70 hours and washed as above.

(c) As for treatment (b), but 100% of dry sulphuryl chloride was used.

Treatments (b) and (c) resulted in yellow and light-brown wool respectively, but weight losses were less than 0.2% in both cases.

(v) Ammonium thioglycollate treatment [Farnworth (1961)]:

Air-dry wool was treated at 40°C for 30 minutes in

1% (v/v) thioglycollic acid + 6% (v/v) 0.880 ammonia solution in absolute ethanol (20:1 liquor:wool ratio), then washed in ethanol. Weight change was not recorded.

(vi) Potassium hydroxide treatments [see, e.g., Freney and Lipson (1940): McPhee (1959)]:

Three separate lots of air-dry wool were treated at 50:1 liquor:wool ratio for 5 minutes at 20°C in 1M (5.6%) potassium hydroxide dissolved in -

(a) 96% ethanol,

(b) saturated aqueous potassium chloride,

(c) distilled water,

then neutralised in 0.01N sulphuric acid. Weight losses were (a) 1.6%, (b) 1.2%, and (c) 1.5%.

(vii) Potassium tert-butoxide treatment:

Vacuum-dry wool was heated under reflux for 3 hours in 1M potassium tert-butoxide in tert-butanol (100:1 liquor: wool ratio) then washed with tert-butanol and ethanol. Weight change was less than 0.2%. The potassium tert-butoxide solution was prepared by heating anhydrous tert-butanol with the appropriate amount of clean dry potassium metal.

(viii) Solvent extractions:

Solvent extraction treatments were carried out using a range of redistilled solvents in a jacketed Soxhlet extractor, thus allowing the temperature to be maintained

at the desired value. The various extractions were as follows -

(a) Ethanol, 20°C, 5 hours - weight loss 0.7%.

(b) Ethanol, 70°C, 5 hours - weight loss 1.2%.

(c) Ethanol, 70°C, 100 hours - weight loss 1.3%.

(d) Ethanol, 100°C, 24 hours - weight change not recorded.

(e) Anhydrous tert-butanol, 70°C, 5 hours - weight change less than 0.2%.

(f) 98-100% formic acid, 70°C, 5 hours - weight loss 10.0%.

(g) Formic acid extractions were also carried out by treating wool for 1 hour at 50:1 liquor-wool ratio and various temperatures in the range 0-100°C. Weight changes for these treatments will be given in figure 2.12 (page 39).

The 100°C ethanol extraction (d) was carried out on a few mgm of wool previously extracted with cold ethanol, by heating in a sealed evacuated glass tube. The tert-butanol extraction (e) was done under completely anhydrous conditions to restrict solvent action to the outer surface of the fibres [Bradbury and Leeder (1963)]; Anderson and Leeder (1965a,b)].

(ix) Peptide hydrolysis [Leach et al. (1964)]:

Wool was heated at 100°C for 20 hours in 0.01N

hydrochloric acid at 200:1 liquor:wool ratio. Weight loss was 12.5%.

(x) Ammonium hydroxide treatment:

Wool was immersed in 0.880 ammonia solution at 20°C for 24 hours and 200:1 liquor:wool ratio. Weight loss was 1.3%.

(xi) Mercuric acetate treatment [Leeder (1965)]:

Wool was treated at 20°C for 50 hours in 0.1M mercuric acetate dissolved in 0.1N acetic acid, at 100:1 liquor:wool ratio. Weight increase was 38%.

(xii) Conversion of cystine to lanthionine [Cuthbertson and Phillips (1945); Crewther et al. (1967)]:

Wool was treated at 60°C for 20 hours with 1% aqueous potassium cyanide at 100:1 liquor:wool ratio. Weight loss was 10.2%.

(xiii) Oxidation with peracetic acid [Alexander and Earland (1950); Thompson and O'Donnell (1959)]:

Oxidation of cystine to cysteic acid was carried out by immersing wool in 90% aqueous acetic acid containing 5% (v/v) peracetic acid, at 100:1 liquor:wool ratio and 20°C for 48 hours. Weight increase was 0.5%.

(xiv) Pronase (enzymatic) digestion [Springell (1963)]:

2 gm wool was treated with 20 mgm pronase [K and K laboratories] in 400 ml pH8 ammonium acetate/ammonia buffer (containing 10% (v/v) ethanol as bactericide) at 37°C for 2 weeks. Weight loss was 7.7%.

(xv) Hyaluronidase (enzymatic) digestion:

0.2 gm wool was treated with 2 mgm hyaluronidase [Sigma Chemical Company] in 40 ml pH 8.4 borate buffer, at 40°C for 9 weeks. Weight change was not recorded.

(xvi) Staining with phosphotungstic acid [Bones and Sikorski (1967)]:

Wool was treated at 40°C and 100:1 liquor:wool ratio for 18 hours with 1% (w/v) solution of phosphotungstic acid in 50% aqueous ethanol. Weight change was not measured.

(xvii) Supercontraction treatment [see e.g., Crewther et al. (1967)]:

Wool was heated at 100°C for 1 hour (100:1 liquor: wool ratio) in 8M aqueous lithium bromide solution. Weight loss was 2.2%.

(xviii) Stretching of wool:

Studies on stretched wool were made by mounting single fibres between a fixed clamp and a movable clamp to which a screw mechanism was attached. This stretching frame was constructed so that it could be placed on the microscope stage, allowing continuous observation during extension of the fibre.

(xix) Greasy wool:

Fleece wool, containing approximately 50% of contaminants such as wool grease, dirt and suint salts, was used without further treatment.

(xx) Tip wool:

The (degraded) tip third of the original Merino 64's staples was cleaned as described earlier for the base portions, then ethanol-extracted at 70°C for 5 hours. Weight loss due to the ethanol-extraction was 1.1%.

(xxi) Powdered wool:

Untreated wool was reduced to a macroscopic powder by grinding in a mortar and pestle under liquid nitrogen.

(xxii) Abraded wool:

(a) Wool was abraded by treating wool, cut to approximately 2 mm lengths, with an aqueous suspension of fine carborundum powder in a Vibromix high-frequency agitator for 36 hours at room temperature.

(b) Single fibres were rubbed firmly between thumb and forefinger.

(xxiii)Worsted fabric:

Some studies were also made on a typical Merino 64's plain-weave worsted fabric, undyed and unfinished. The fibres comprising such a fabric would have been subjected to the normal mechanical processing operations such as combing, gilling, spinning, weaving, etc.

Following all chemical treatments, the modified wool was washed for at least one week in twice-daily changes of deionised distilled water. Weight changes were measured as

the change in vacuum-dry weight (10^{-4}mm Hg, 20°C-20 hours or 100°C-1 hour) and, where possible, by drying and weighing the extract. Weight changes of less than 0.2% have not been recorded - possible loss of short fibres, finely dispersed material and soluble material during treatments and washings will produce errors of this order in the dry weights.

[E] RESULTS AND DISCUSSION:

2[E](i) Conformation of Epicuticle on Merino Wool:

The results which follow concern the physical structure of epicuticle on the surfaces of Merino 64's fibres. Section 2[E](ii) will relate these results to a wider range of keratin fibres.

A necessary experiment to begin a study of the association between epicuticle and single cuticle cells is to isolate cuticle cells and add chlorine water. This was originally attempted by Hock et al. (1941). The isolation procedure should preferably result in minimal chemical and physical damage. Bradbury and co-workers (1965a, 1966, 1967) have found that the treatment which involves the least chemical degradation consists of dissolving or modifying the intercuticular cementing material with 98-100% formic acid and separation of the loosened scales by severe mechanical agitation. Unfortunately, cuticle material

prepared by this method did not develop Allworden sacs when treated with chlorine water. This observation was also made by King (1967). The reason for this, as shown by microscopic examination, was that the isolated cuticle consisted of cracked and broken cell fragments; very few pieces were large enough to represent a complete cuticle cell.

During a search for mechanically-milder separation techniques, it was found that partial detachment of cuticle cells occurred if fibres were treated without agitation in formic acid at elevated temperatures. The effect is shown in figure 2.4 for a fibre which had been extracted in formic acid at 70°C for 5 hours. The degree of separation of the scales is considerably enhanced by subsequently immersing the fibre in chlorine water (figure 2.5). Note particularly that sac formation occurs on these partially-detached scales.

Complete detachment of a small number of cuticle cells was achieved by heating wool, in the absence of mechanical agitation, in formic acid for 1 hour at 100°C. When these samples were washed free of acid and chlorine water added, many of the single cuticle cells formed sacs similar in appearance to those which form on the surface of untreated fibres. Figure 2.6 shows a typical result. The difference in optical density between the sac + contents and the cuticlecell proper, was readily confirmed by 'through focussing' over the depth of the scale. Sacs again appeared

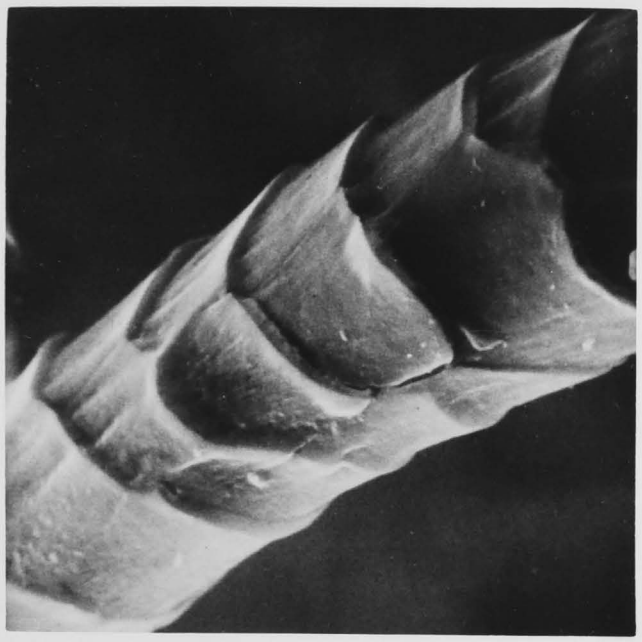


Figure 2.4. Scanning Electron Micrograph of Merino Fibre Treated with HCOOH,70°C,5 hrs.

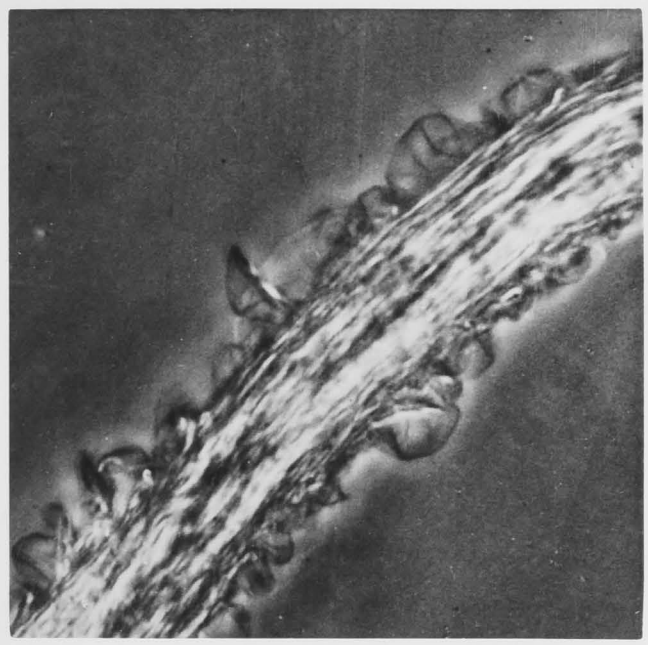


Figure 2.5. Effect of Chlorine Water on Merino Fibre Treated with HCOOH,70°C,5 hrs.

Figure 2.6. Effect of Chlorine Water on an Isolated Cuticle Cell.

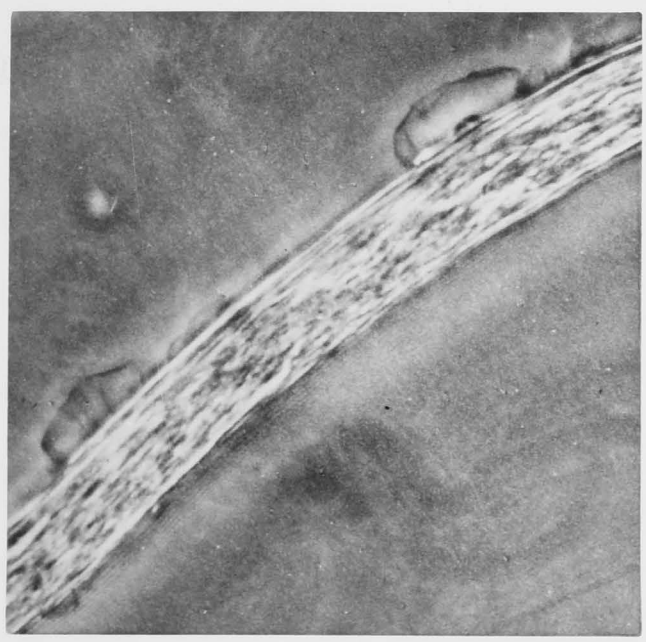


Figure 2.7. Effect of Chlorine Water on Fibre 'Descaled' by Two-stage HCl-HCOOH Treatment.

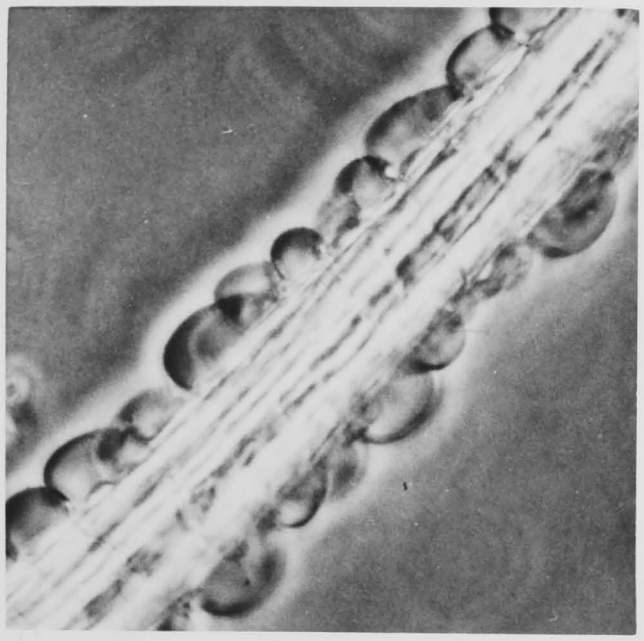
on partially-detached scales, giving a similar effect to that shown in figure 2.5.

Comparable observations were made when bromine water was used in place of chlorine water.

Figure 2.7 shows a fibre after partial hydrolysis with 0.01N hydrochloric acid at 100°C for 20 hours, followed by treatment with formic acid at 100°C for 1 hour. This treatment was found to induce separation of large intact sheets of cuticle material, resulting in almost completely descaled fibres. Note, however, that the few remaining cuticle cells give a positive Allworden reaction, again indicating association of epicuticle with individual cuticle cells, and at the same time demonstrating the chemical inertness of the membrane.

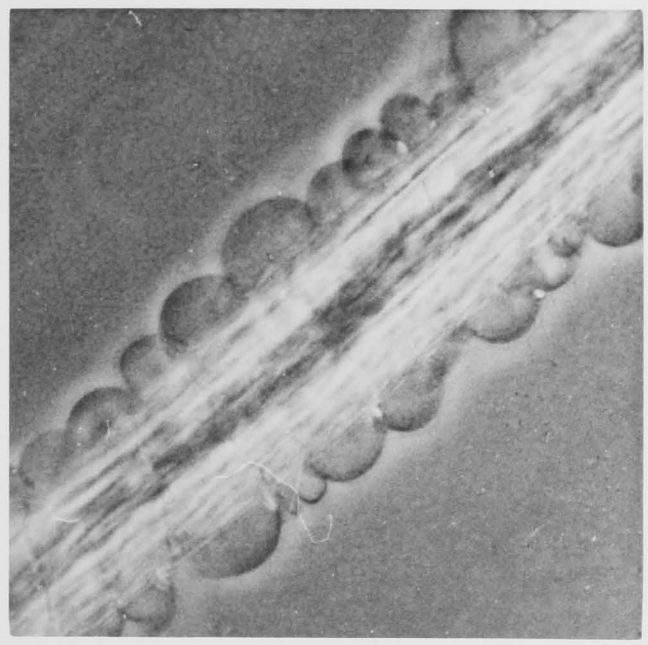
To demonstrate that the sacs which form on fibres and isolated cuticle cells after pre-treatment with hot formic acid are genuine Allworden sacs, a series of treatments ranging from 5 minutes at 0°C up to 1 hour at 100°C were applied to Merino wool samples. The effects of chlorine water on some of these wools are shown in figures 2.8 - 2.11, while weight loss as a function of treatment-temperature is summarised in figure 2.12.

Although considerable dissolution and dispersion of material occurred, it is obvious that the swellings produced by chlorine water after pretreatment for 1 hour in formic



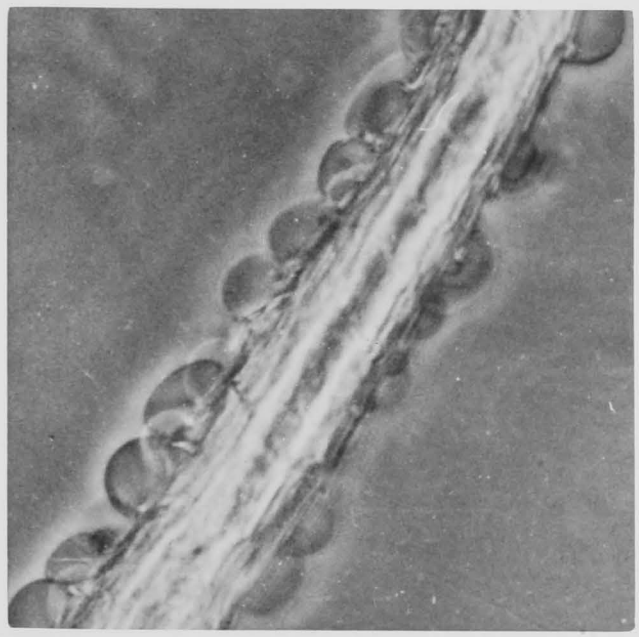
20μ

Figure 2.8. Effect of Chlorine Water on Fibre Pretreated with HCOOH,0°C,5 min.



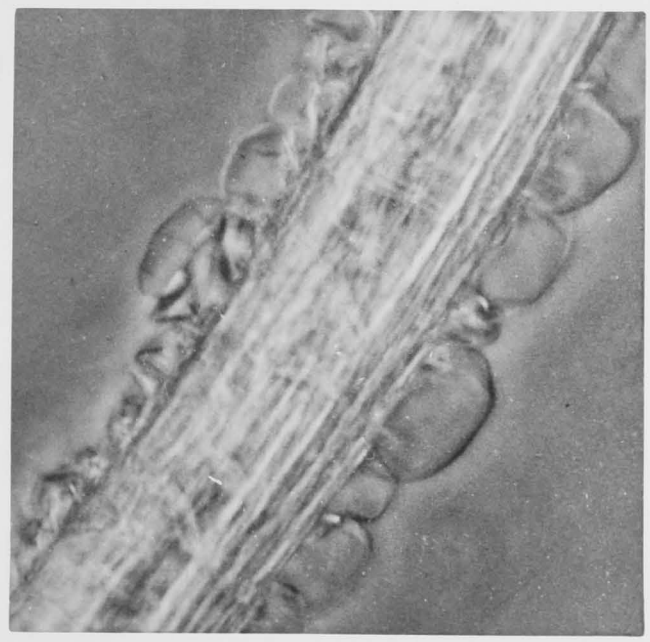
20μ

Figure 2.9. Effect of Chlorine Water on Fibre Pretreated with HCOOH,20°C,1 hr.



20μ

Figure 2.10. Effect of Chlorine Water on Fibre Pretreated with HCOOH,60°C,1 hr.



20μ

Figure 2.11. Effect of Chlorine Water on Fibre Pretreated with HCOOH,100°C,1 hr.

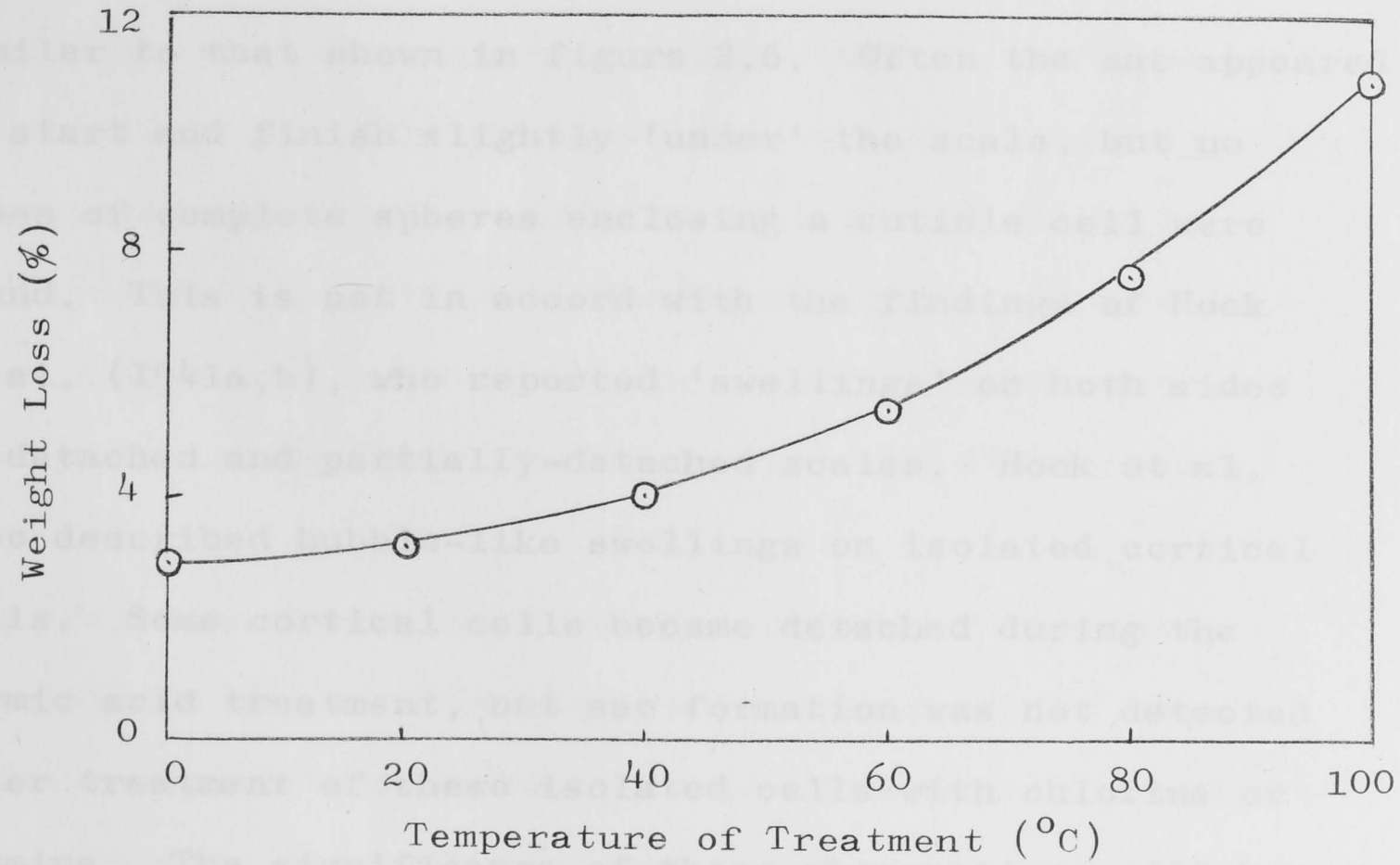


Figure 2.12. Material Dissolved or Dispersed from Merino Wool by Treatment for 1 hour in Formic Acid.

acid at 100°C are Allworden sacs and not side-effects of the formic acid treatment. A gradual transition from smooth 'tight' sacs to slightly-wrinkled sacs occurs as the temperature of pretreatment increases - this probably results from partial balancing of the osmotic pressure by chlorine-soluble material dissolved from the (degraded) inner regions of the fibres.

Note the detached cuticle cell and associated Allworden sac in figure 2.11.

Most sacs on isolated scales were geometrically similar to that shown in figure 2.6. Often the sac appeared to start and finish slightly 'under' the scale, but no cases of complete spheres enclosing a cuticle cell were found. This is not in accord with the findings of Hock et al. (1941a,b), who reported 'swellings' on both sides of detached and partially-detached scales. Hock et al. also described bubble-like swellings on isolated cortical cells. Some cortical cells became detached during the formic acid treatment, but sac formation was not detected after treatment of these isolated cells with chlorine or bromine. The significance of these observations will be discussed later in this chapter.

The present demonstration of epicuticle membranes associated with single scales agrees well with the conclusions of Hock et al. (1941a,b), who claimed to see similar sacs but unfortunately did not publish photographic proof. Lindberg (1949) acknowledged the observations of Hock and co-workers, but his faith in the continuous membrane concept was not shaken, so he stated that "this could be due to osmotic pressure in the nuclear hollows of the scales and should not be confused with the Allworden reaction."

It is hereby suggested that the sacs formed on isolated scales <u>should be</u> 'confused' with the Allworden reaction. They have the same optical and geometric

characteristics; they can be observed to form and grow in the same manner; they can be collapsed by the addition of salt solutions as with the Allworden sacs on untreated wool; and occasionally, during addition of chlorine water to fibres pretreated with formic acid, a scale + sac (as in figure 2.6) can be seen to detach itself from the parent fibre (such as the fibre in figure 2.5) and float freely in the solution.

Because of the controversial nature of the above statement and the far-reaching consequences of the non-continuous epicuticle concept, it is necessary to reconsider every argument, and look again at every experimental result, which have led to the almost-universal acceptance of the theory that epicuticle is a continuous external sheath.

Mercer (1953) summarised most of the work done prior to 1953, and listed 7 main points which, taken together, 'prove' epicuticle to be continuous; each of these points will be considered in turn -

(a) "<u>Allworden bubbles can be seen to cover several scales</u>" - in most cases, each sac seems to cover only one scale outline, but sometimes a large sac appears to cover several scales, and occasionally the formation of this large sac out of smaller ones can be observed [see, e.g., Lindberg (1949)]. The opposite view was taken by Hock et al. (1941a), who wrote, "In a few cases two or more foci of swelling

occurred on a single scale and were observed to flow together to form one large sac. It was also evident that the size and shape of the sacs were associated with similar properties of the scales."

Further, Muller (1939) suggested that the two-dimensional overlapping of scales occasionally resulted in sacs appearing to span more than one scale. The limited depth of focus of the optical microscope can lead to ambiguous interpretations in cases such as this.

More recently, Kassenbeck (1958) resolved this difference - he examined longitudinal sections of wool fibres and found that one or two 'ribs' or false scale edges occurred approximately mid-way along the length of many cuticle cells.

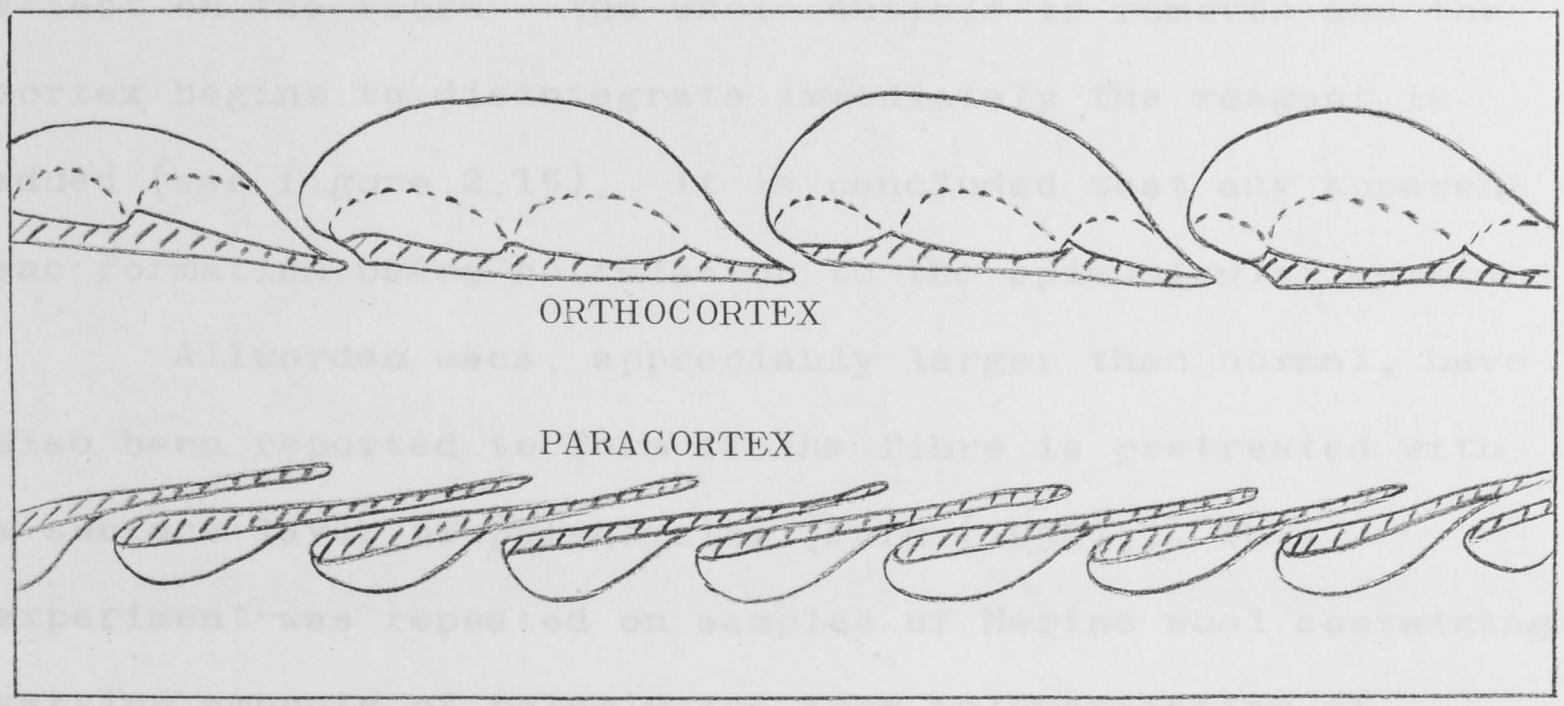


Figure 2.13. Variation of Allworden Reaction with Shape of Cuticle Cell in Fine Merino Wool [Kassenbeck (1958)].

Figure 2.13 represents Kassenbeck's interpretation of the reported observations that sacs covered several scales. This led him to conclude that epicuticle surrounds the individual cuticle cells. A cuticle fragment showing a typical false scale edge is shown in figure 2.14. Kassenbeck's electron micrographs indicated a distinction between cuticle cells surrounding the paracortex and those surrounding the orthocortex - discussion of the possible existence of ortho- and paracuticle cells is given in the next section of this thesis.

King and Bradbury (1967) reported that large sacs covering several scales were produced by treatment of wool with chlorosulphonic acid. Re-examination of this reaction showed that chlorosulphonic acid has a violently disruptive effect on the fibre - the whole cuticle is removed and the cortex begins to disintegrate immediately the reagent is added (see figure 2.15). It is concluded that any apparent sac formation bears no relation to the epicuticular membrane.

Allworden sacs, appreciably larger than normal, have also been reported to form if the fibre is pretreated with a surface layer of polyglycine [Ford (1952)]. This experiment was repeated on samples of Merino wool containing varying amounts of polyglycine from polymerisation of anhydrocarboxyglycine [Bradbury and Shaw (1960)] and thiazolid-2:5-dione [Bradbury and Leeder (1960)]. Sac

Figure 2.14. Cuticle Cell Fragment Showing False Scale Edge (Electron Micrograph, from Bradbury and Chapman (1964)).

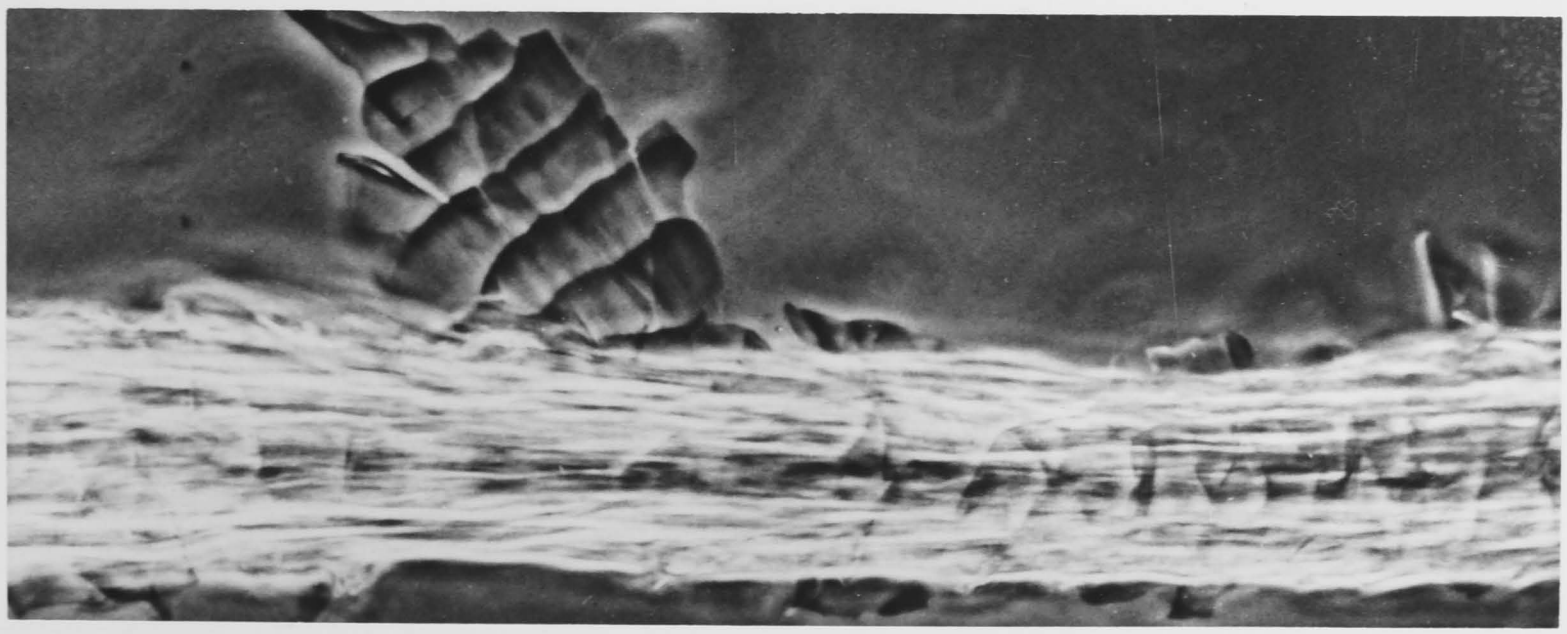


Figure 2.15. Merino Fibre Treated with Chlorosulphonic Acid.

formation appeared to be slightly retarded by the surface layer of polymer. Polymer fragments could be seen on the surface of the raised membrane, but sacs larger than those which form on untreated fibres were not observed.

(b) "Isolation of sheets of epicuticle large enough to cover several scale faces" [Mercer et al. (1949)] - Examination of the many published photomicrographs of isolated epicuticle fragments shows that they always occur as thin sheets which are rolled-up to varying extents, making area measurements extremely difficult and uncertain. In the present work, the maximum length of each fragment was measured from enlarged photomicrographs and compared with the dimensions of intact Allworden sacs.

Epicuticle material was isolated by shaking chlorine-treated wool in water. The resulting suspension was concentrated by centrifugation to give the preparation shown in figure 2.16. The length of these fragments varied between 10μ and 55μ, with an average length of 30μ. This type of measurement was originally made by Zahn (1952), who found a maximum length of 40μ and an average length of 25-30μ. This led him to the conclusion that epicuticle was associated with the individual scales.

When wool fibres were stretched after immersion in chlorine water, epicuticle fragments became detached from the fibre surface and were left suspended as thin rolled-up

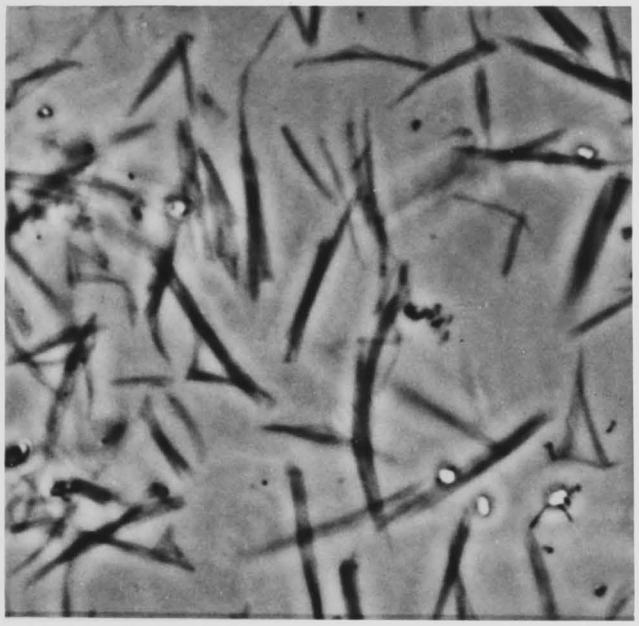


Figure 2.16. Epicuticle Shaken from Chlorine-treated Merino Fibres.

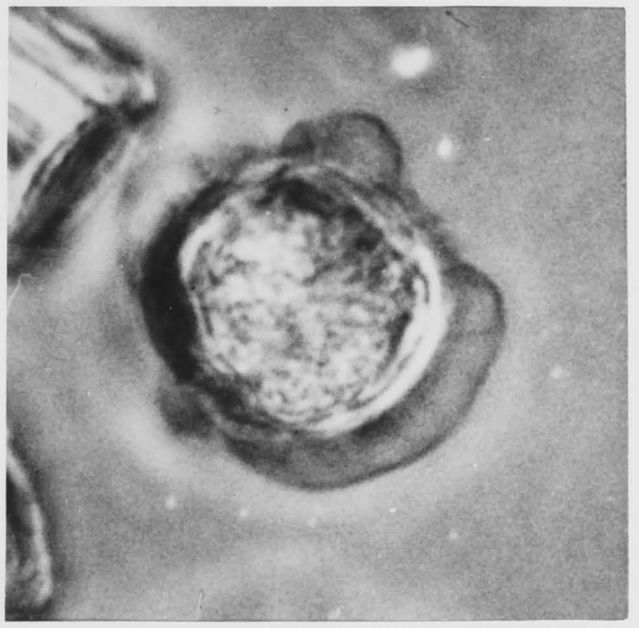


Figure 2.17. Cross-section of Merino Fibre, Treated with Chlorine Water.

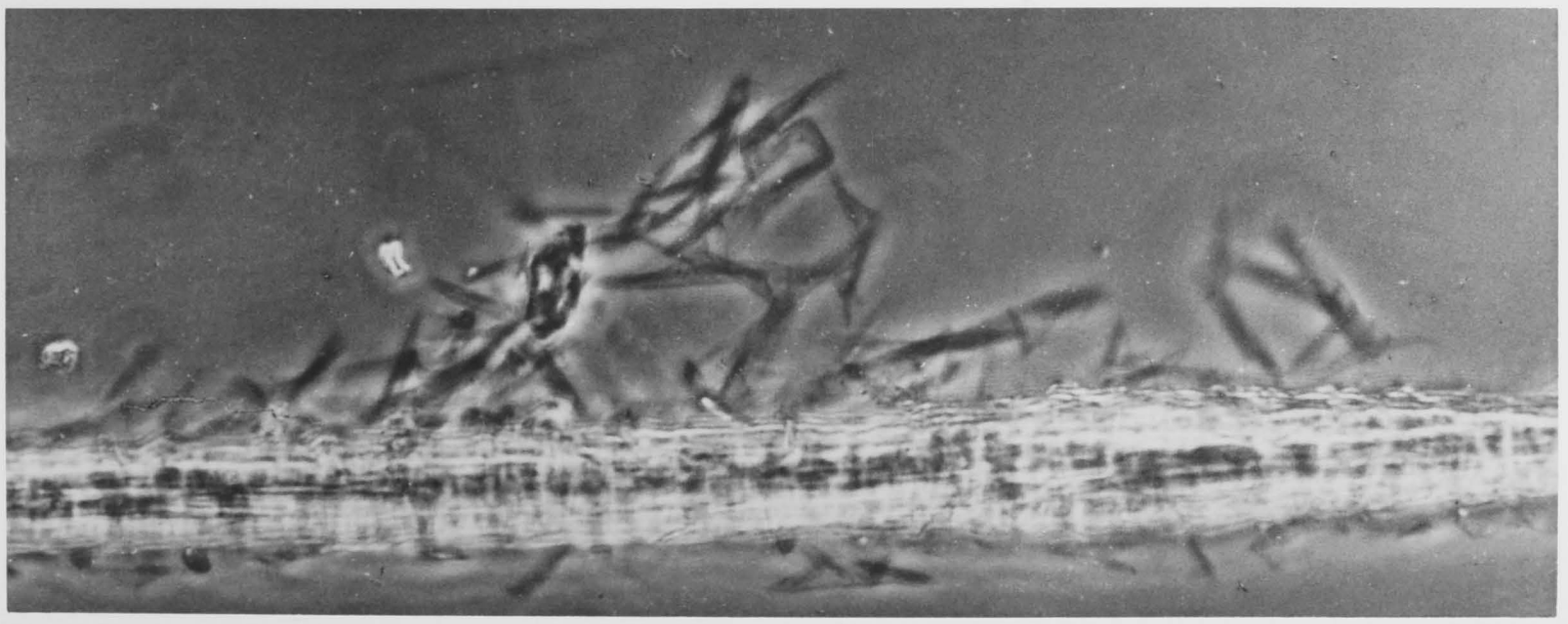


Figure 2.18. Epicuticle Detached by Stretching a Merino Fibre in Chlorine Water.

sheets (figure 2.18). These fragments were also found to vary between 10μ and 50μ in length. The perimeters of intact Allworden sacs produced on fibre cross-sections had similar dimensions, e.g. the perimeter of the larger sac shown in figure 2.17 is approximately 50μ, as is the perimeter of the sac on the isolated cuticle cell shown in figure 2.6. These measurements show that epicuticle fragments have the approximate maximum dimensions of intact Allworden sacs, each of which covers only one cuticle cell.

The difficulty in correlating dimensions of epicuticle fragments with scale length may have arisen from lack of appreciation of the two-dimensional surface coverage of the Allworden sacs (i.e. transverse and longitudinal). During attempts to isolate cuticle cells without physical damage, it was found that treatment of fibres with 0.01N hydrochloric acid at 100°C for 20 hours followed by formic acid at 100°C for 1 hour resulted in separation of large sheets of intact cuticle material. This treatment therefore constitutes a method for direct microscopical examination of the scale structure of fibres, free from the focussing difficulty encountered with intact cylindrical fibres.

Two such preparations are shown in figures 2.19 and 2.20. It can be seen that the Allworden sacs will be, in most cases, longer in the transverse direction than in the longitudinal direction (40-60μ compared with 10-15μ).

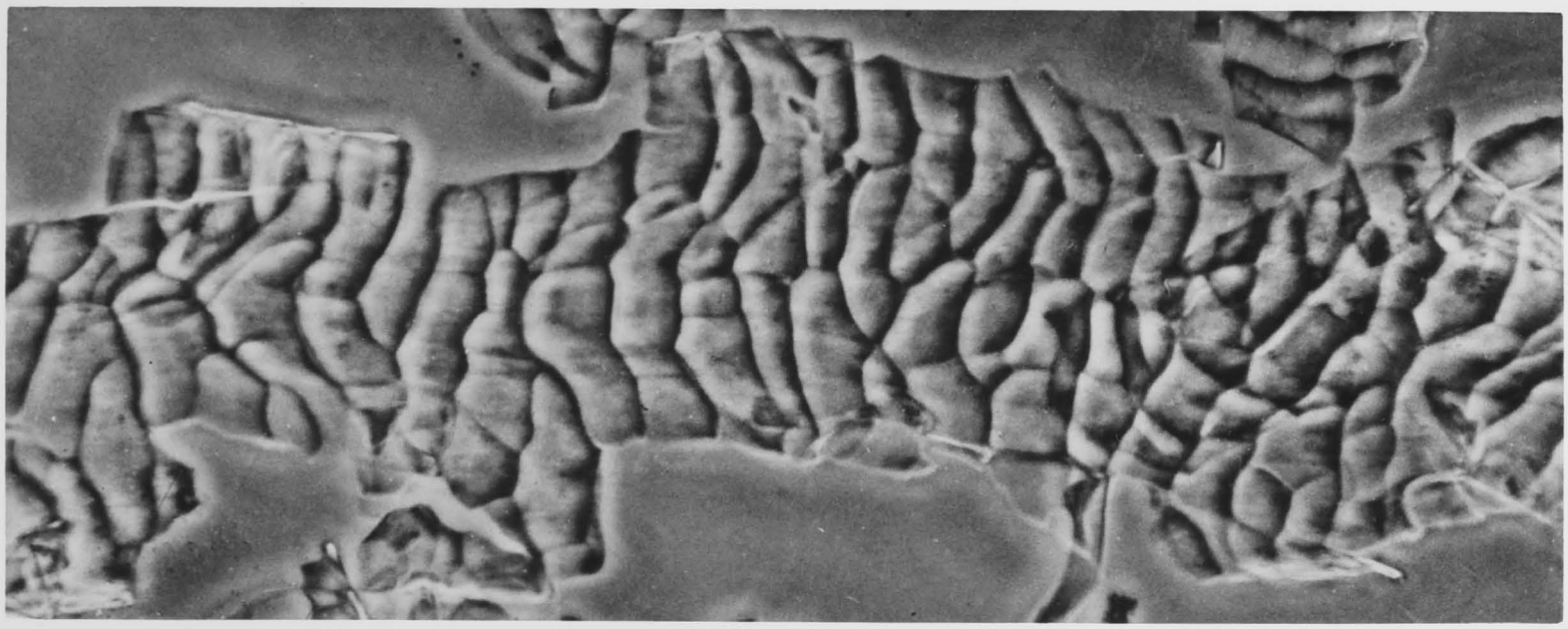


Figure 2.19.

20μ

Sheets of Merino Cuticle Material, Isolated by Two-stage HCl-HCOOH Treatment

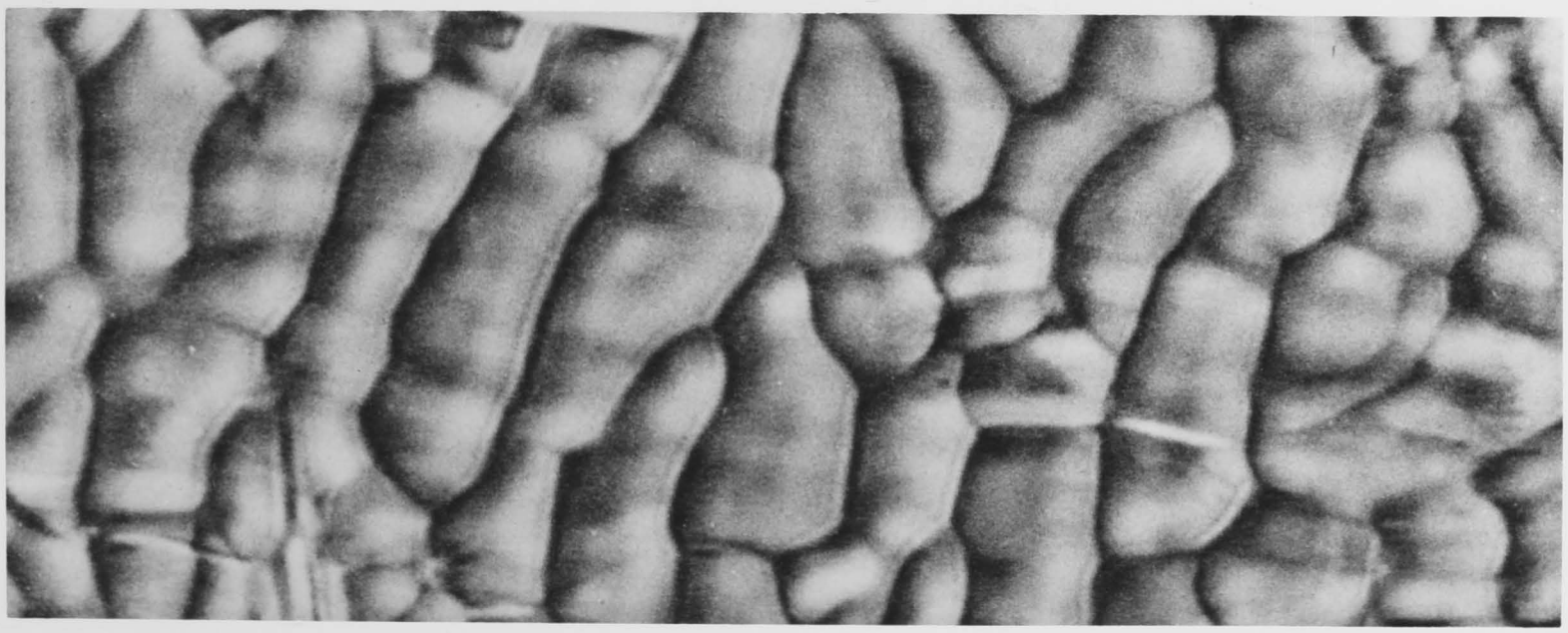


Figure 2.20.

10μ

Thus isolated epicuticle fragments could be as much as 3 times longer than the exposed length of the cuticle cell in the longitudinal direction. Failure to realise this fact may have misled earlier workers to the conclusion that detached pieces of epicuticle were larger than the individual cuticle cells.

(c) "Separation of continuous tubules consisting of the surface layers of cuticle when the cortex etc. is dissolved" [Mercer (1953); Mercer and Golden (1953a); Leveau et al. (1953a)] - Mercer and Golden observed large sacs which slowly coalesced to form a continuous outer sheath when oxidised fibres were placed in 1% aqueous sodium bicarbonate. The effect was hastened when stronger alkalies were used. These authors interpreted this as a demonstration of continuous epicuticle, following the earlier work of Alexander and Earland (1950), who found that fibres oxidised with peracetic acid quickly dissolved in dilute ammonia, leaving a 'pheripheral resistant membrane'.

Parisot and Leveau (1953) did not agree with Mercer and Golden's interpretation. The membranes appeared to be very thick when compared with that normally observed for epicuticle and the sacs showed evidence of scale edges. They suggested that the sacs in question were bounded by the entire cuticle; this was consistent with earlier observations on the action of bromine water on some animal fibres [Leveau et al. (1952)].

It should also be mentioned here that the present author could not reproduce Mercer and Golden's sac effect on peracetic-oxidised wool, in agreement with Fraser and Rogers (1955a) and King and Bradbury (1967).

An alternative explanation for the origin of peracetic-alkali swellings will be given in sub-section (g).

Another report of continuous surface tubules resulting from dissolving the bulk of the fibre is that of Leveau et al. (1953a). By completely avoiding agitation during digestion of wool in aqueous sodium sulphide, these workers isolated tubular membranes "having the size of several scales and showing the characteristics of epicuticle". When the experiment was repeated during the present work, the result shown in figure 2.21 was obtained - this compares well with the photographs of Leveau et al. However, when the residue was washed in 98-100% formic acid as part of the present author's standard procedure (see chapter 3), the 'continuous tubules' dispersed into a suspension of discrete membranes (epicuticle?) (see figure 2.22). This effect will be considered in greater detail in the work on whole-fibre cell membranes (chapter 3). Apparently the formic acid removes the last traces of intercellular cementing material, allowing separation of the individual cell residues.

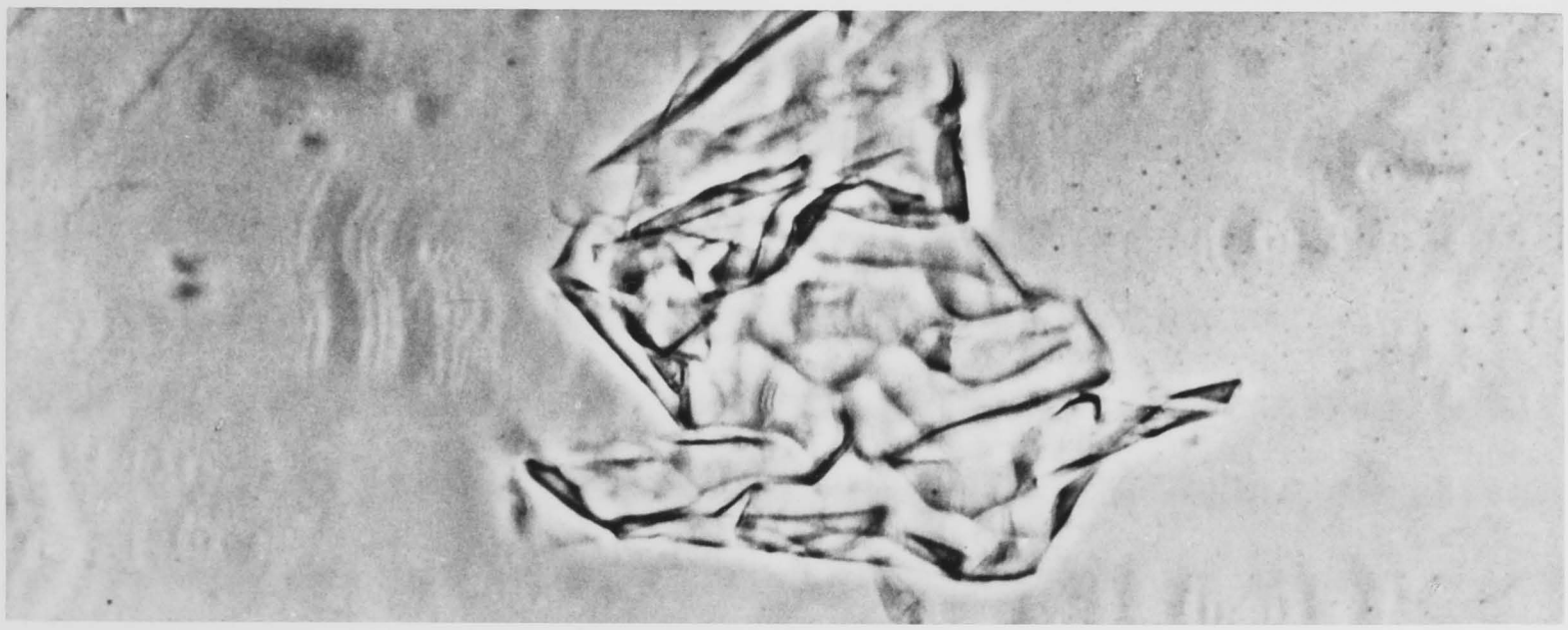


Figure 2.21. Residual Membranes from Digestion of Wool in Aqueous Sodium Sulphide.

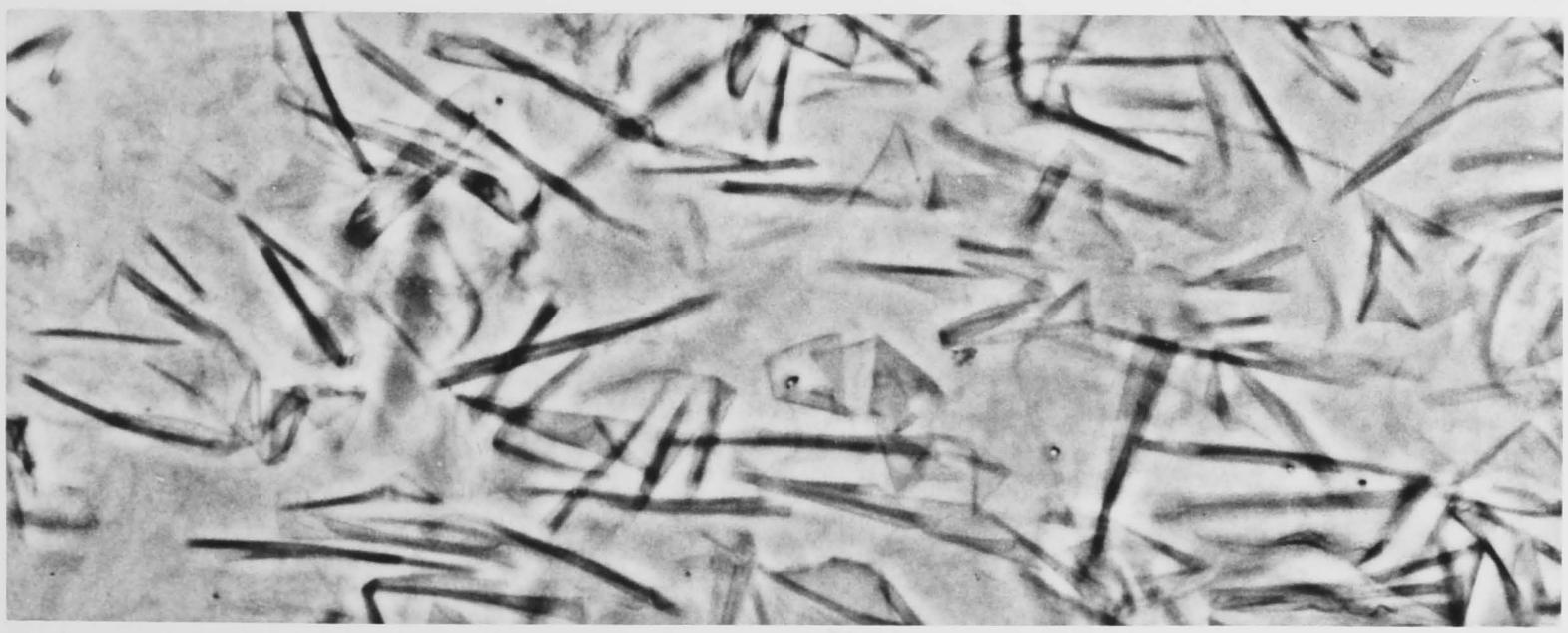


Figure 2.22. Residual Membranes from Sodium Sulphide Digestion, After Washing in Formic Acid.

(d) "The separation of a continuous sheath when supercontracted fibres are digested for several days in trypsin" [Mercer et al. (1949)] - The sheaths so isolated were described by the authors to consist of epicuticle + exocuticle. More recently, electron microscopy of longitudinal sections of wool fibres [e.g. Kassenbeck (1958); Dobb et al. (1961)] has clearly established that exocuticle is not continuous, but is a component of the individual cuticle cells. It seems much more likely that the phenol-trypsin treatment used by Mercer et al. resulted in a membranous residue similar to that shown in figures 2.21 and 2.22. Again, this type of preparation will be discussed further in chapter 3.

Lagermalm (1954) published many electron-micrographs of membranes isolated or loosened by tryptic digestion of supercontracted wool. Numerous ridges, streaks and folds occur in the surface membranes. Some of these folds could represent an artefact of the drying-out which occurs in the evacuated specimen chamber of the electron microscope, while others probably result from overlaps of epicuticle from various single cuticle cells. In fact, Lagermalm states that these ridges are associated with or related to the scale edges, but concludes that epicuticle is a continuous membrane covering more than one scale surface.

Mercer et al. (1949) prepared similar sheaths by

tryptic digestion of reduced and alkylated fibres [after Geiger and Harris (1942)]. They concluded that the ridges observed in this preparation gave to the complete sheath the appearance of scale edges when viewed optically. It is now suggested that these ridges <u>are</u> the original scale edges, and are formed by the epicuticular membrane associated with the individual scales.

The further suggestion is made, that electron micrographs of vacuum-dried fibrous residues can lead to ambiguous interpretations, in the absence of parallel observations of aqueous suspensions under the light microscope.

(e) "<u>The epicuticle-like sheath of whale hair, which lacks a normal cuticle</u>" - a sample of this material was not available for study, but, if whale hair lacks a normal cuticle, it is an anomalous case, so comparisons between the surface components of this fibre and those of other animal hairs should not be made. Presumably, if there is no scale structure, Allworden sacs do not form - and it must be remembered that Lindberg et al. (1949) originally defined epicuticle as the membrane which forms the Allworden sacs when wool fibres are treated with chlorine water.

Resistant membranes, which appear to be similar to the epicuticle of wool when viewed under the optical- or electron-microscope, have been isolated from the surfaces

of human skin and fingernails [Lagermalm et al. (1951)] and feathers [Philip et al. (1950, 1951)], but again these structures do not give a positive Allworden reaction.

(f) "The (epicuticular) sheath extends beyond the point at which a normal bubble reaction on individual scales can be elicited in the growing hair in the follicle" [Mercer (1951)]: - Many workers have noted that Allworden sacs can be formed below the original skin surface on plucked fibres, and that the level at which this formation starts is the level in the follicle where hardening or keratinisation begins [Hock et al. (1941a,b); Mercer (1949); Schuringa et al. (1952a,c)]. This is the point at which cysteine is converted to cystine (i.e. free thiol groups to di-sulphide cross-links). It will be shown later in this chapter that the presence of cystine is necessary for sac formation to occur, so lack of a positive Allworden reaction at lower levels in the follicle simply means that this prerequisite has not been met. If epicuticle is, in fact, of cell membrane origin, it will still be present in the lower levels of the follicle, but in a non-keratinised form.

(g) "The lifting-off of a continuous membrane when a fibre exhibiting a 'normal' chlorine-induced bubble reaction is transferred to an alkaline medium" - This reaction has been reported by Muller (1939), Millson and Turl (1951), Mercer and Golden (1953a), and Millson (1955) for alkali

on chlorine-treated fibres, and by Schuringa et al. (1953) for alkali on bromine-treated fibres. A typical example is shown in figure 2.23 (untreated wool was reacted with chlorine water in the usual manner, then the chlorine water was washed away by drawing 0.1% aqueous potassium hydroxide under the microscope cover slip by means of filter paper). The exact shape of this apparent membrane was found to vary with time of treatment and with strength and concentration of alkali. The effect obtained suggests that epicuticle is a continuous outer membrane, and this is probably the most widely quoted single piece of evidence in favour of the continuity concept.

However, the following new experimental evidence nullifies the above interpretation. If fibres from which epicuticle has been removed by immersion and agitation in chlorine water and which therefore give no Allworden reaction (figure 2.24), are subjected to the same sequence of operations, the result depicted in figure 2.25 is obtained. Clearly, this effect cannot be used as an argument in favour of continuous epicuticle. Significantly, Muller (1939) was the first to observe the 'chlorine + alkali' continuous membrane on untreated wool, yet he still concluded that epicuticle was associated with the individual scales. The most likely explanation is that the alkali swells and dissolves the strongly-oxidised and degraded fibre 'from the outside in', resulting in a gelatinous mass of degraded

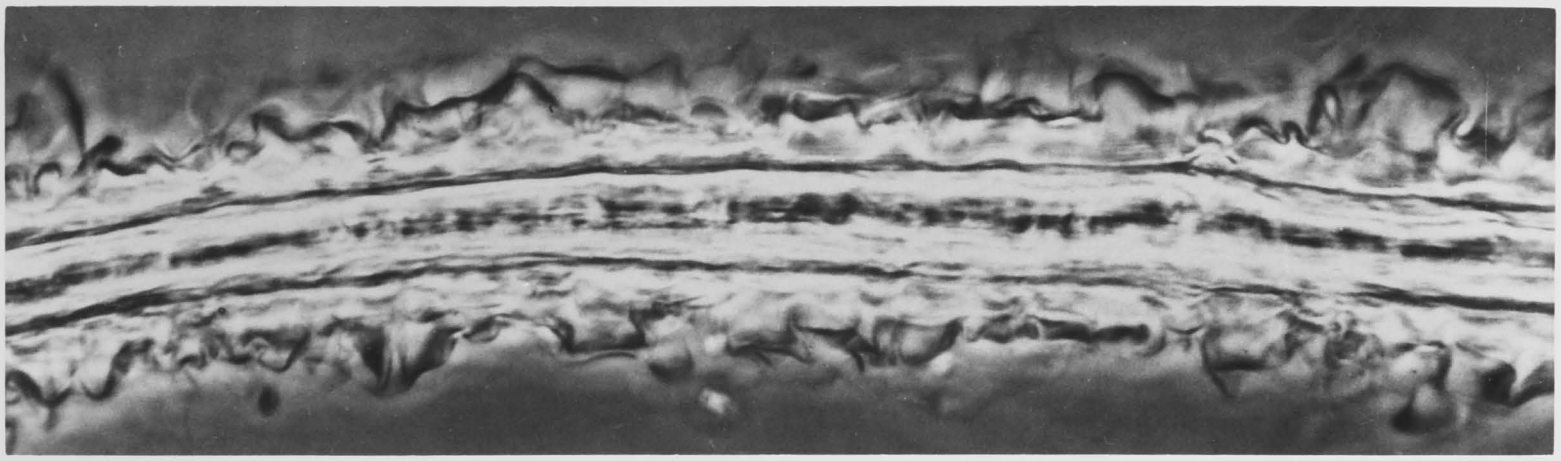


Figure 2.23. Effect of Chlorine Water Followed by Dilute Potassium Hydroxide.

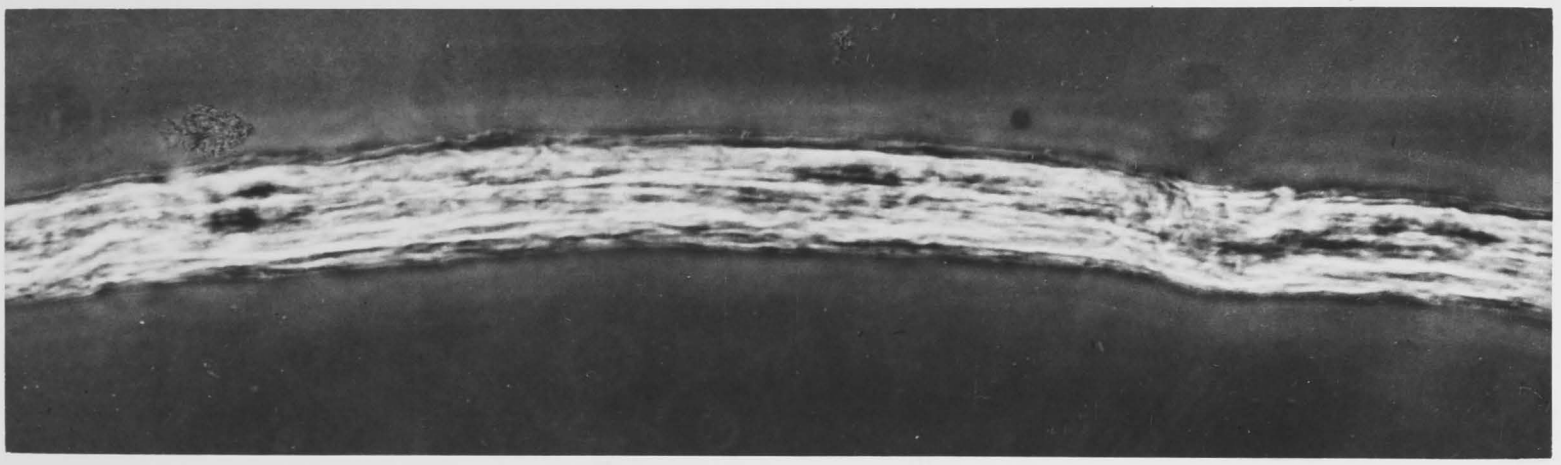


Figure 2.24. Effect of Chlorine Water on Merino Fibre Devoid of Epicuticle.

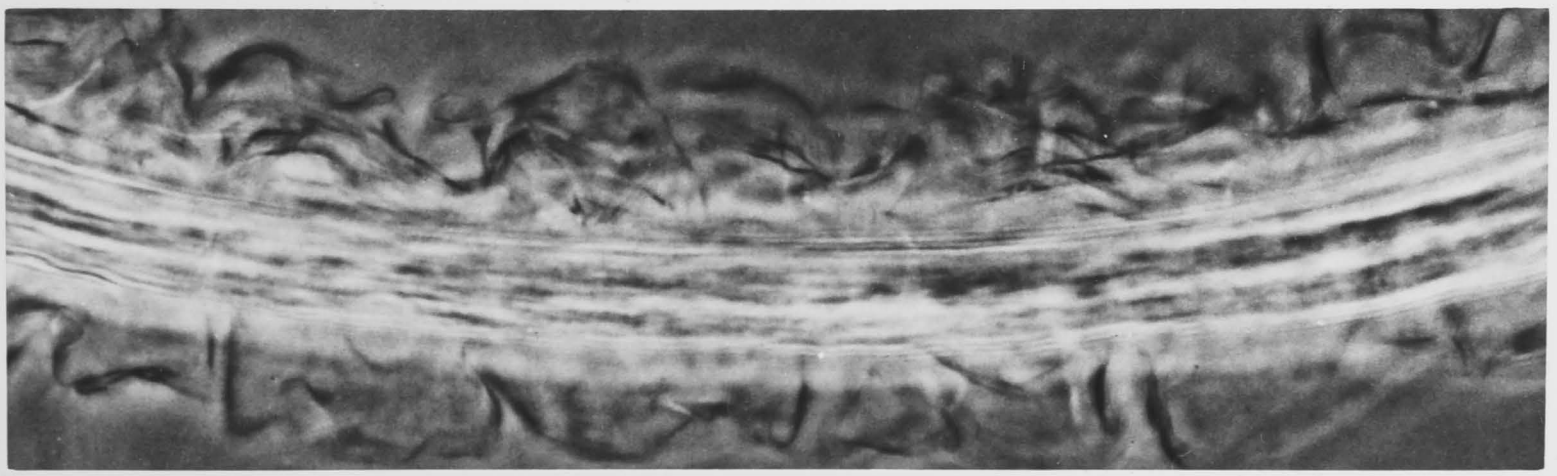


Figure 2.25. Effect of Chlorine Water Followed by Dilute Potassium Hydroxide on Fibre Devoid of Epicuticle.

protein material. With increasing time and/or increasing strength of alkali the fibre eventually passes into solution. This is best demonstrated by using higher concentrations of alkali, e.g. 1M potassium hydroxide, when the chlorine-treated fibres swell greatly and dissolve completely in less than one minute.

Thus King (1967) found that even chemically-resistant epicuticle is soluble in dilute alkali after the chlorine pretreatment, presumably because chlorine breaks stabilising disulphide cross-links by oxidation to cysteic acid and other cystine oxides.

Similar effects to those shown in figures 2.23 and 2.25 have also been observed on cross-sections of fibres.

In addition to a range of alkalies at various concentrations, several other protein-swelling reagents were applied to chlorine-pretreated fibres. The surface-dissolution effect was obtained with 8M urea at pH 10, formic acid, hydrochloric acid, sulphuric acid, and even with 'mild' protein solvents such as formamide and 50% (v/v) aqueous n-propanol, and again the presence or absence of epicuticle did not appear to affect the results obtained. In particular, note that the reaction can proceed in the presence of 8M urea, demonstrating that the effect is not due to osmotic forces under a semi-permeable membrane, as with the Allworden reaction - Allworden sacs collapse on the addition of concentrated salt solutions!

Photomicrographs depicting various stages of the reaction between chlorine-pretreated wool and 8M urea at pH 10 (figure 2.26), formic acid (figure 2.27), formamide (figure 2.28) and aqueous n-propanol (figure 2.29) are representative of the effects obtained.

In view of the above results, it now seems likely that the large bubbles and continuous membranes observed when fibres oxidised with peracetic acid are treated with dilute alkalies [Mercer and Golden (1953a)], are formed by a similar process.

The only remaining observations favouring continuity of epicuticle are those concerned with diffusion of dyes into the fibre. Whewell and Woods (1944) found that the rate of uptake of Methylene Blue was much greater after application of mechanical pretreatments, which were therefore considered to disrupt an external membranous barrier to diffusion. An alternative explanation for this effect will be given in chapter 4.

Millson and Turl (1950) claimed to observe an unbroken film overlying the edge of a scale when a fibre was knotted so that the scales became distended. However, they then negated their claim by showing that the knotted region of the fibre exhibited greatly increased dye uptake.

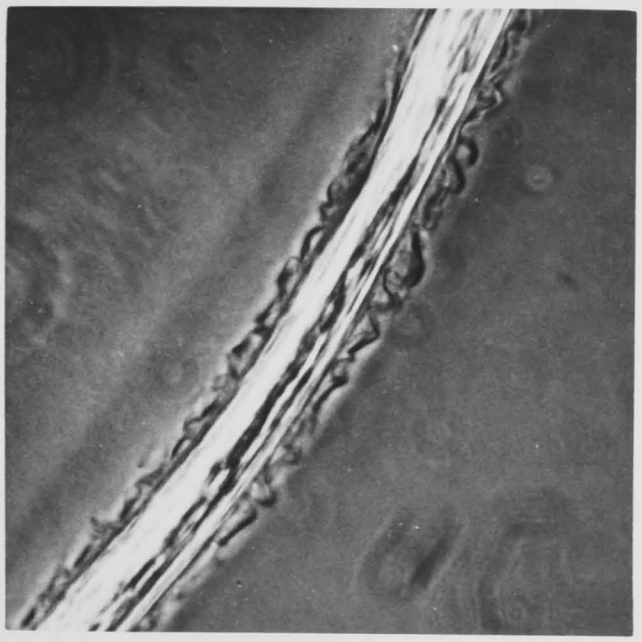


Figure 2.26. Effect of 8M Urea (pH 10) on Chlorine-treated Fibre.

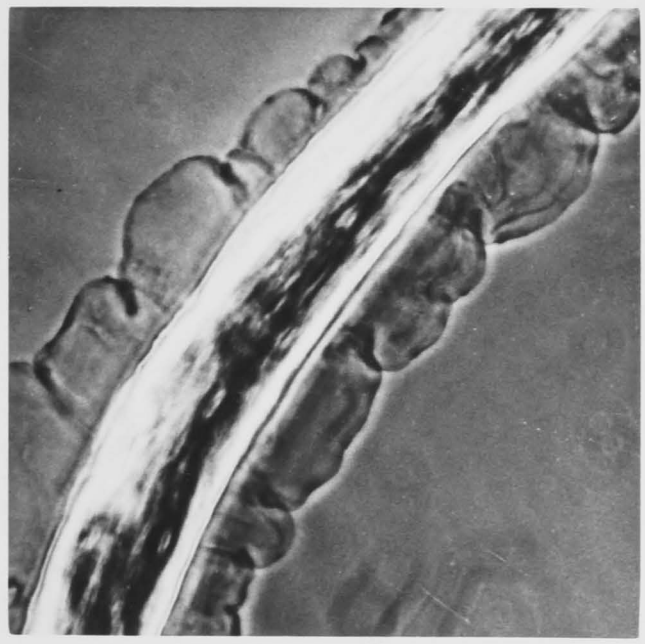


Figure 2.27. Effect of Formic Acid on Chlorine-treated Fibre.

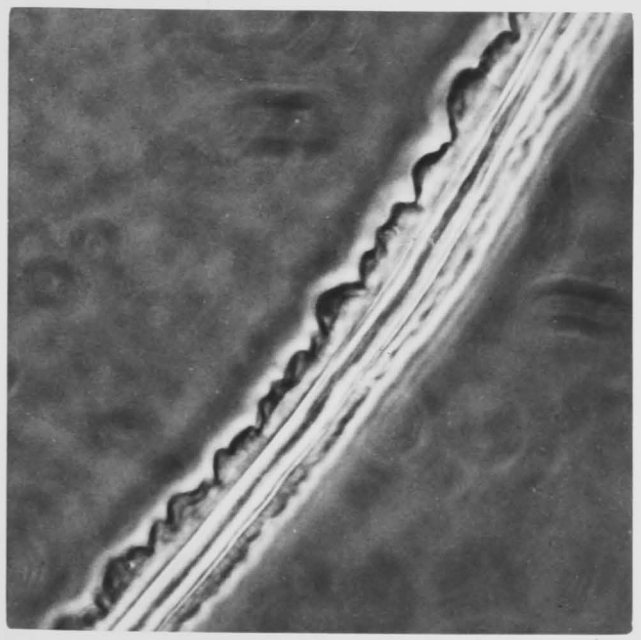


Figure 2.28. Effect of Formamide on Chlorine-treated Fibre.

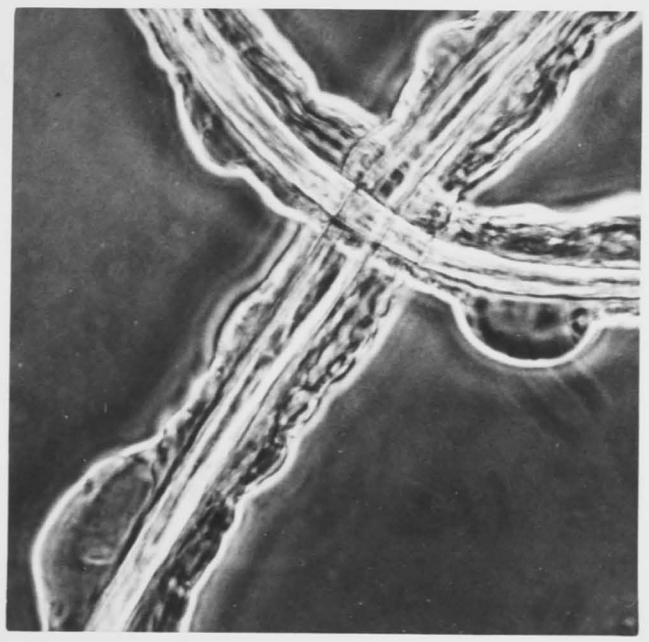


Figure 2.29. Effect of Aqueous Propanol on Chlorine-treated Fibre.

It is pertinent to conclude this section by summarising the observations which influenced the various proponents of discontinuous epicuticle -

Muller (1939) - from careful light-microscopy of Allworden sac formation on modified and unmodified keratin fibres;

Hock, Ramsay and Harris (1941a,b) - from sac formation on partially- and fully-detached cuticle cells;

Roberts (1945) - from distension and swelling of scales when a fibre was placed in cuprammonium hydroxide solution;

Zahn (1952) - from length measurements of isolated epicuticle fragments;

O'Reilly, Whitwell, Steele and Wakelin (1952) - from indications that dye uptake was influenced more by the degree of disturbance of the scales than by the presence of epicuticle;

Kassenbeck (1958) - from electron-microscopical observations of the presence of false scale edges.

Published separately, these observations apparently made insufficient impact to be universally accepted, and thus necessitated the detailed appraisal given in this section.

The foregoing results and arguments favour, very strongly indeed, the concept that epicuticle covers each

individual scale, in contrast to the current belief that it forms a continuous external sheath. The remainder of this thesis is largely devoted to a re-evaluation of some of the chemical and physical properties of wool and other keratin fibres which are supposedly influenced by the presence of a continuous external membrane. In the work which follows, many more pieces of evidence, direct and indirect, will be presented in support of the discontinuous epicuticle concept.

2[E](ii) Relation Between Epicuticle and Scale Structure:

Definite association between epicuticle and scale structure was shown by the results and discussion in the preceding section, where, as with the majority of earlier investigations, fine Merino fibres were used as the substrate for Allworden sac formation.

The surface structures of mammalian hair and fur fibres show considerable variations between different animals, and between different fibre types from a particular animal [see, e.g., Hardy and Plitt (1940); Wildman (1955); Kassenbeck (1959)]. Considerable changes in scale arrangement are also found along the lengths of some classes of individual fibres [Hausman (1920); Hardy and Plitt (1940); Lyne and McMahon (1951); Wildman (1955)] although this fact is not generally appreciated [Wildman (1955)].

The present section is concerned with studies of the relation between Allworden sac pattern and scale geometry, using both intact fibres and isolated cuticle material.

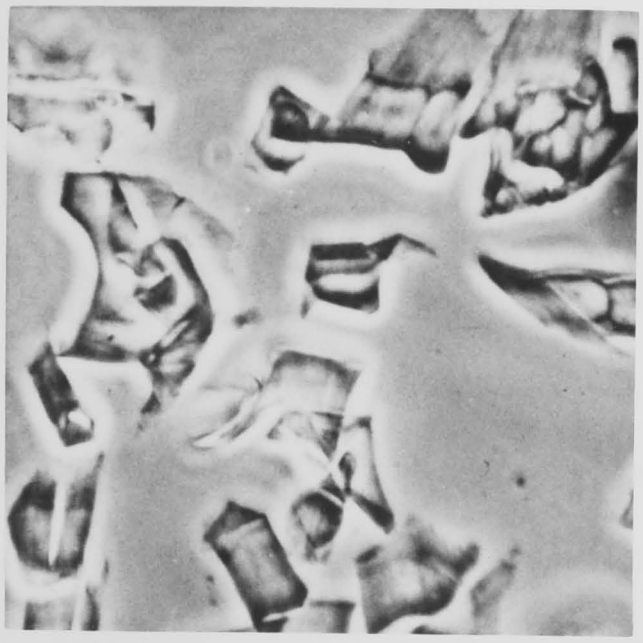
The techniques described in section (i)(b) for isolation of individual cuticle cells (treatment for 1 hour at 100°C in 98-100% formic acid) and for separation of intact sheets of cuticle material (treatment for 20 hours at 100°C in 0.01N hydrochloric acid followed by 1 hour at 100°C in 98-100% formic acid) were employed to enable direct microscopic examination of scale shape and arrangement. In the text and figures which follow, these methods are referred to as 'HCOOH-treatment' and 'two-stage HCl-HCOOH treatment' respectively. Results of microscopical examinations provide much of the experimental data, and a large number of photomicrographs are presented to illustrate the observed effects. Scanning electron micrographs are referred to as 'Stereoscans', while all other photomicrographs are optical micrographs taken under phase contrast.

(a) Merino 64's fibres:

The relation between epicuticle and scale structure for Merino 64's fibres was described in section 2[E](i) and thoroughly illustrated by figures 2.1 - 2.29. In the present section, the earlier results are summarised and new data presented, in order to permit comparisons with other fibres.

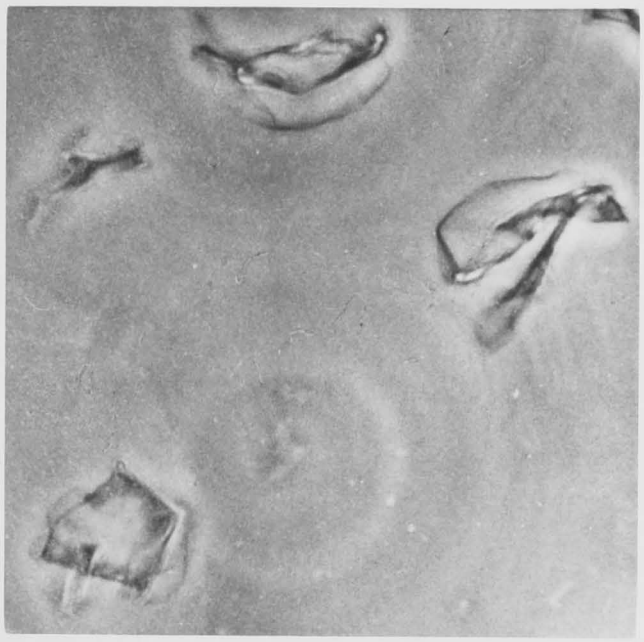
Figure 2.30 shows a typical preparation of cuticle material following HCOOH-treatment, while figure 2.31 shows further examples of sac formation on individual cuticle cells. Note that one scale is lying almost flat, yet the inflated membrane is still visible. Because of the restricted space between microscope slide and cover slip, most cuticle cells assumed this position. Allworden sacs were observed on only a small percentage of the scale fragments - most pieces were smaller in area than that expected for an intact cuticle cell (see discussion in section (i)(b)).

The <u>exposed</u> length of a cuticle cell, when expressed as the distance between successive scale edges, is of the order of 10-15μ, while the length of fibre surface covered by Allworden sacs varies from 10μ to 30μ - compare, e.g., figures 2.1 and 2.2, page 14a. The difference in maximum length is explained by the presence of false scale edges on some cuticle cells. The scanning electron micrograph (figure 2.32) shows a scale fragment which has such a false scale edge, viewed from the <u>underside</u> of the fragment. Measurements on a large number of optical photomicrographs of cuticle sheets and whole fibre profiles gave an average value of 12μ for the distance between successive 'scale edges', while measurements of the length of fibre surface spanned by Allworden sacs gave a value of around 18μ.



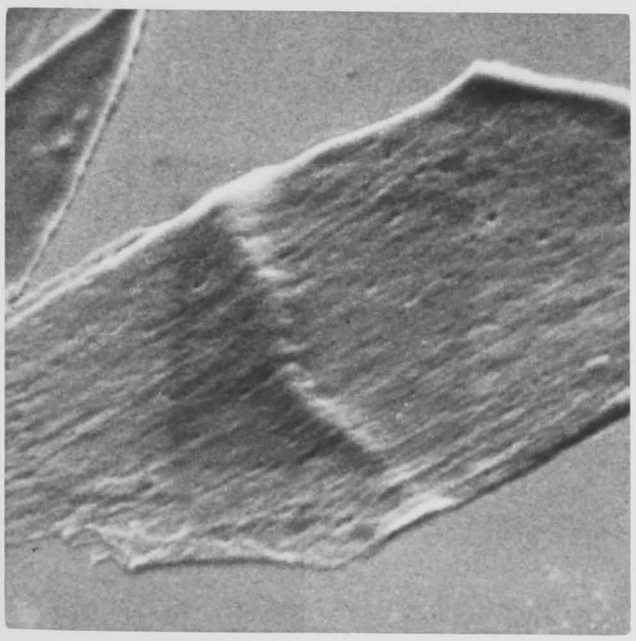
20μ

Figure 2.30. Merino Cuticle Material Isolated by HCOOH-treatment.



20μ

Figure 2.31. Allworden Sacs on Isolated Merino Cuticle Cells.



3μ

Figure 2.32. 'Stereoscan' of a Merino Cuticle Fragment.

6μ

Figure 2.33. 'Stereoscan' of a Merino Fibre.

This shows that approximately every second cuticle cell on Merino 64's wool has a false scale edge.

Further, an average length of 30μ was found for those cuticle cells which gave a positive Allworden reaction, such as those shown in figures 2.6 and 2.31, although many cells were as much as 50-60μ long. These measurements must therefore represent what was originally the transverse dimension of the scale on theintact fibre. A cuticle cell 60μ across would almost completely encircle a fibre of 20μ diameter. Support for this conclusion was obtained by studying Allworden sac formation on fibre cross-sections. Figure 2.17, page 44a, depicts a sac encircling approximately half of the fibre circumference, and occasionally sacs were observed to almost completely encircle the section. Note that many of the scales on the fibre shown in figure 2.33 give the impression of being coronal. Wildman (1955) assumes an average of 2 scales per circumference of Merino 64's fibres.

The transverse dimensions of cuticle cells, as indicated by the scale markings on cuticle sheets - see figures 2.19 and 2.20 (page 45a) - serve to confirm the above observations.

There will also be an increase in sac dimensions following detachment of cuticle cells from the parent fibre, because the area of epicuticle originally under the next

overlapping cuticle cell will be free to contribute to sac formation. This increase will not be very large for Merino 64's, since the cuticle cells of these fibres have a low degree of overlap. Appleyard and Greville (1950) estimate that approximately five-sixths of each cuticle cell forms the external surface on fine and medium wool fibres, so that only one-sixth is involved in (longitudinal) overlap.

The above observations and conclusions are summarised schematically in figure 2.34.

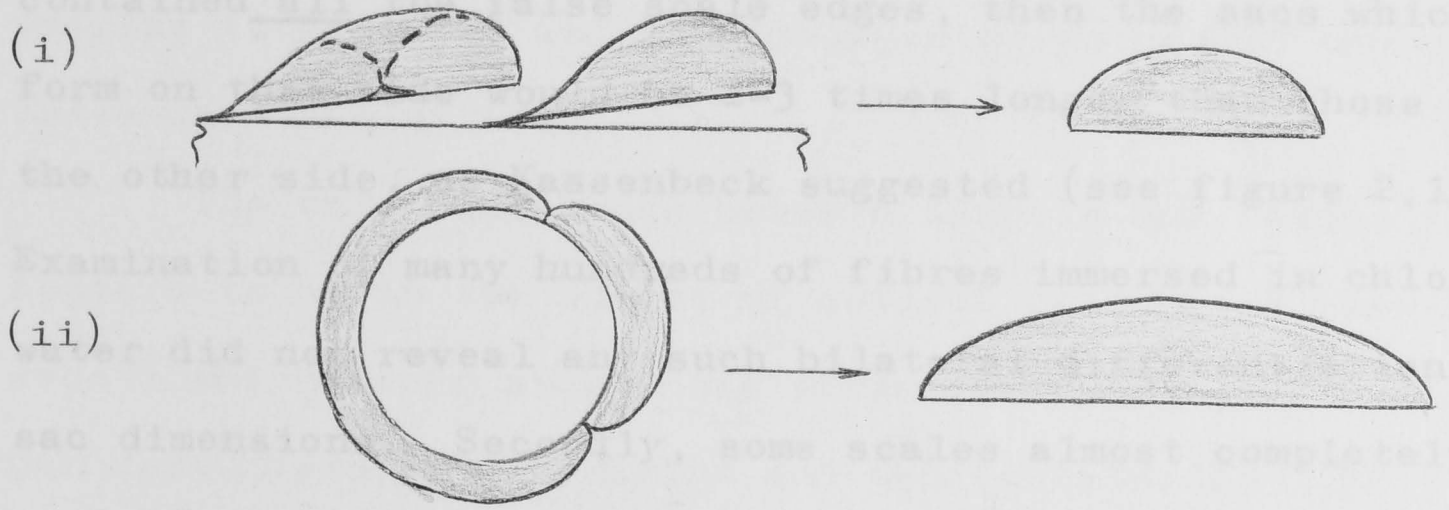


Figure 2.34. Schematic Representation of Formation of Allworden Sacs on Merino 64's Wool, (i) in the Longitudinal Direction, and (ii) in the Transverse Direction.

One further aspect of Merino cuticle structure warrants consideration - Kassenbeck (1958) postulated the occurrence of ortho- and para-cuticle cells, covering the respective ortho- and para-cortical segments of the fibre.

His electron micrographs indicate that false scale edges occur only on the orthocortical side of the fibre, with an associated low degree of scale overlap, while the 'para-cuticle' cells supposedly have a high degree of overlap and are devoid of false scale edges. This was shown in figure 2.13, page 42.

Despite the visual evidence submitted by Kassenbeck, two observations made during the present work reflect on his interpretation. Firstly, if one side of the fibre contained all the false scale edges, then the sacs which form on that side would be 2-3 times longer than those on the other side, as Kassenbeck suggested (see figure 2.13). Examination of many hundreds of fibres immersed in chlorine water did not reveal any such bilateral differentiation in sac dimensions. Secondly, some scales almost completely encircle the fibre - this would involve a change from 'ortho' to 'para' character within a single cell if Kassenbeck's postulate applied.

Kassenbeck's work was done on Merino 110's fibres, which are very fine fibres indeed! Thus, although the present work does not support application of the ortho-para concept to the cuticle cells of Merino 64's wool, the possibility remains that other grades of wool may exhibit bilateral differences in the cuticle.

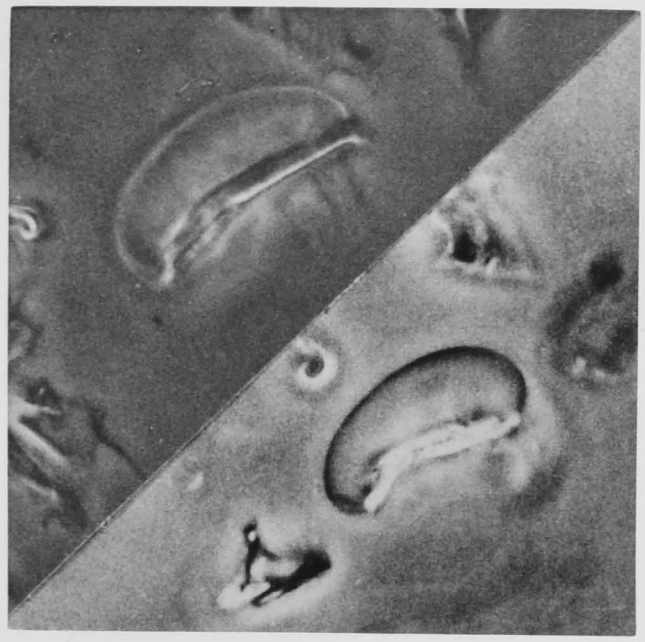
(b) Lincoln 36's fibres:

The diameter of Lincoln 36's fibres (60μ) is approximately 3 times that of Merino 64's fibres (see figure 2.36, but note change in magnification). However, the dimensions of isolated scales and the sacs produced on them (figure 2.35). the Allworden sac pattern (figure 2.36), and the exposed area of cuticle cells (figure 2.37) are all similar to the corresponding dimensions of the finer Merino fibres. Further, Appleyard and Greville (1950) commented on the low degree of overlap (one-sixth) and single-layer structure of the cuticle cells on Lincoln 36's.

The only difference between Lincoln and Merino fibres appears to be in the number of cuticle cells (and therefore sacs?) which occur around the circumferences - 1-2 scales per circumference for Merino and 4-6 for Lincoln. In support of this, figure 2 of Bradbury et al. (1963) is a fibre cross-section from the same sample, showing approximately 6 scale junctions around the circumference. The scanning electron micrograph in figure 2.38 also shows more scale edges across the fibre than with the Merino 64's fibre in figure 2.33.

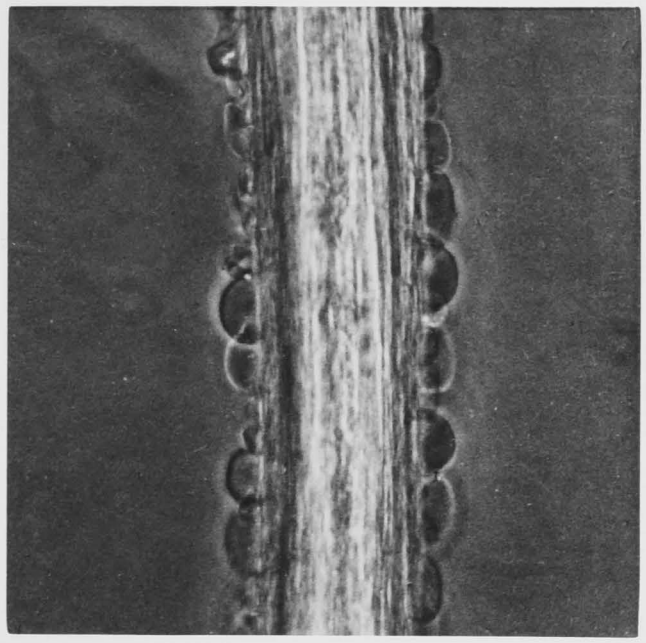
(c) Human hair:

The scale structure of human hair differs from that of Merino and Lincoln wool fibres in that the cuticle cells possess a very high degree of overlap, resulting in a



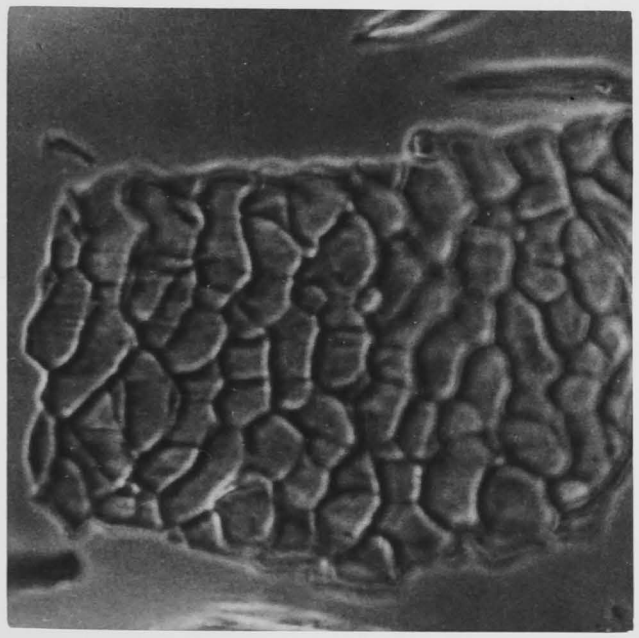
20μ

Figure 2.35. Allworden Sacs on Lincoln Cuticle Cells, Isolated by HCOOH-treatment.



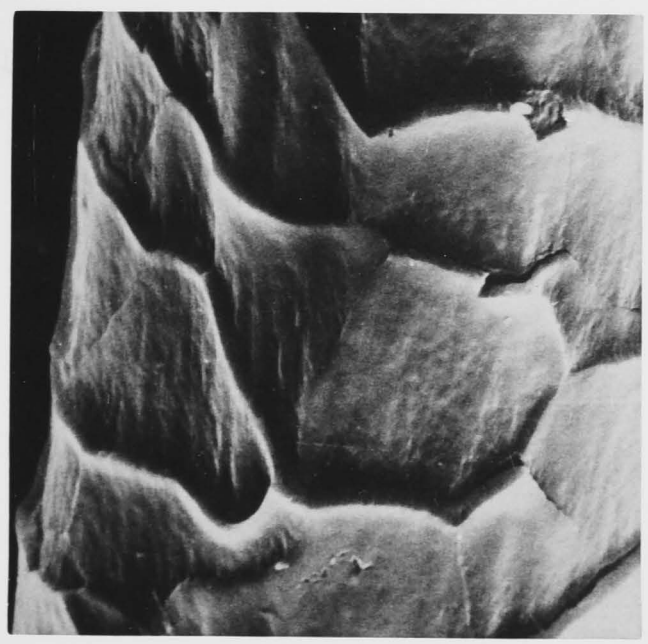
40μ

Figure 2.36. Allworden Sac Formation on Lincoln Fibre.



40μ

Figure 2.37. Lincoln Cuticle Sheet, Isolated by Two-stage HCl-HCOOH Treatment.



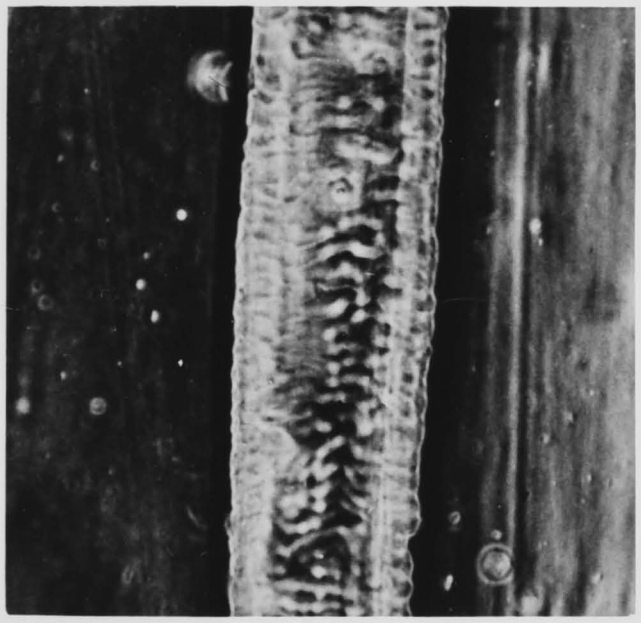
6μ

Figure 2.38. 'Stereoscan' of Lincoln Fibre.

cuticle layer several cells thick [Rudall (1941); Appleyard and Greville (1950)]. This multi-layer structure is typical of most coarse keratin fibres such as animal guard hairs.

Figure 2.39 is an optical micrograph of untreated human hair, mounted on "Sellotape" to increase the amount of surface detail observable [Brown (1959); Bradbury (1961)]; figure 2.40 is a scanning electron micrograph of a similar fibre. The close and regular occurrence of scale edges provides visual evidence for the high degree of overlap. From studies on longitudinal sections, Appleyard and Greville (1950) estimated that only approximately one-sixth of each cuticle cell lies in the outer surface of the fibre, the remainder being under the next several overlapping scales. The distance between successive scale edges, measured on photomicrographs of sheets of cuticle material isolated by the HCl-HCOOH method (see figure 2.41), was found to be around 6μ. This is considerably less than the 12-18μ found for Merino and Lincoln fibres, despite the much greater diameter of human hair - 125μ for the sample used in the present work, compared with 20μ and 60μ respectively for Merino and Lincoln.

Thus the Allworden sac pattern was expected to differ from that for wool, and figure 2.42 shows that this is indeed the case. The entire cuticle seems to have been disturbed by the action of chlorine water, but very few 'typical' Allworden sacs form.

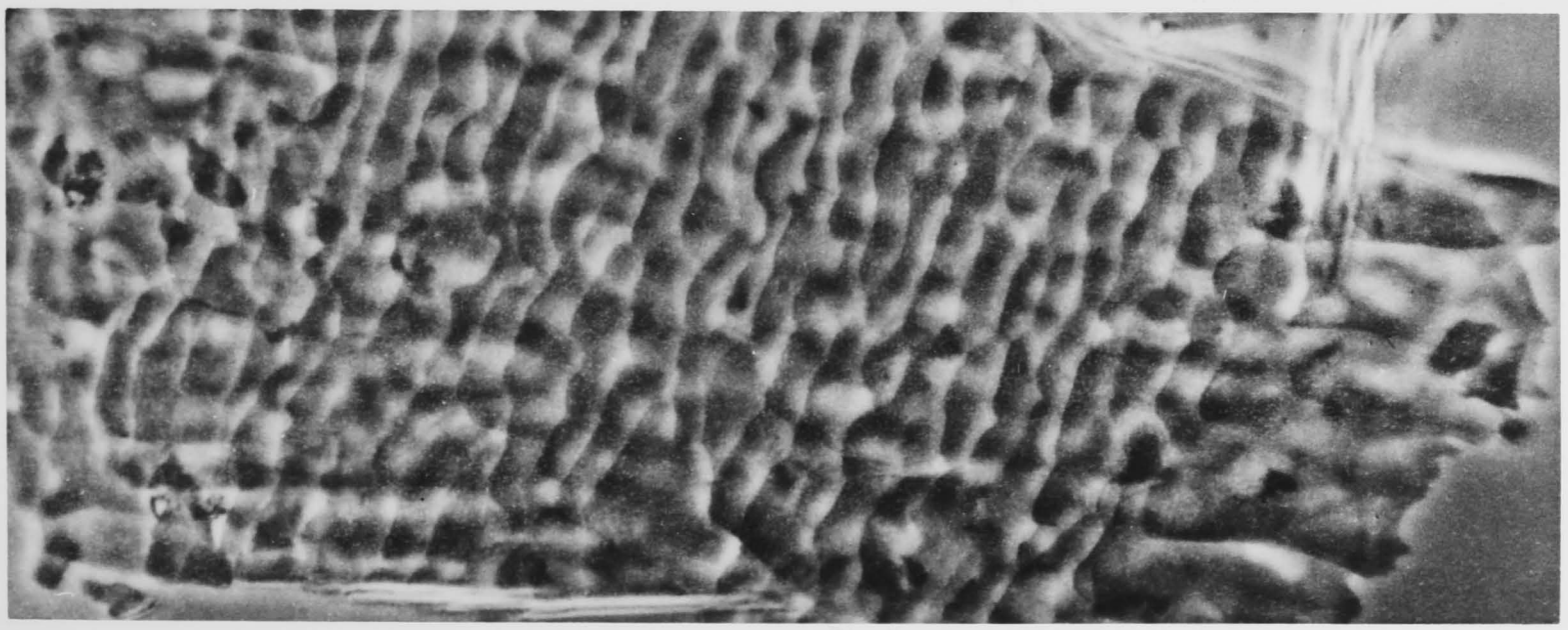


40μ

Figure 2.39. Human Hair, Mounted on 'Sellotape'.

20μ

Figure 2.40. 'Stereoscan' of Human Hair.



20μ

Figure 2.41. Intact Sheet of Human Hair Cuticle, Isolated by Two-stage HCl-HCOOH Treatment.

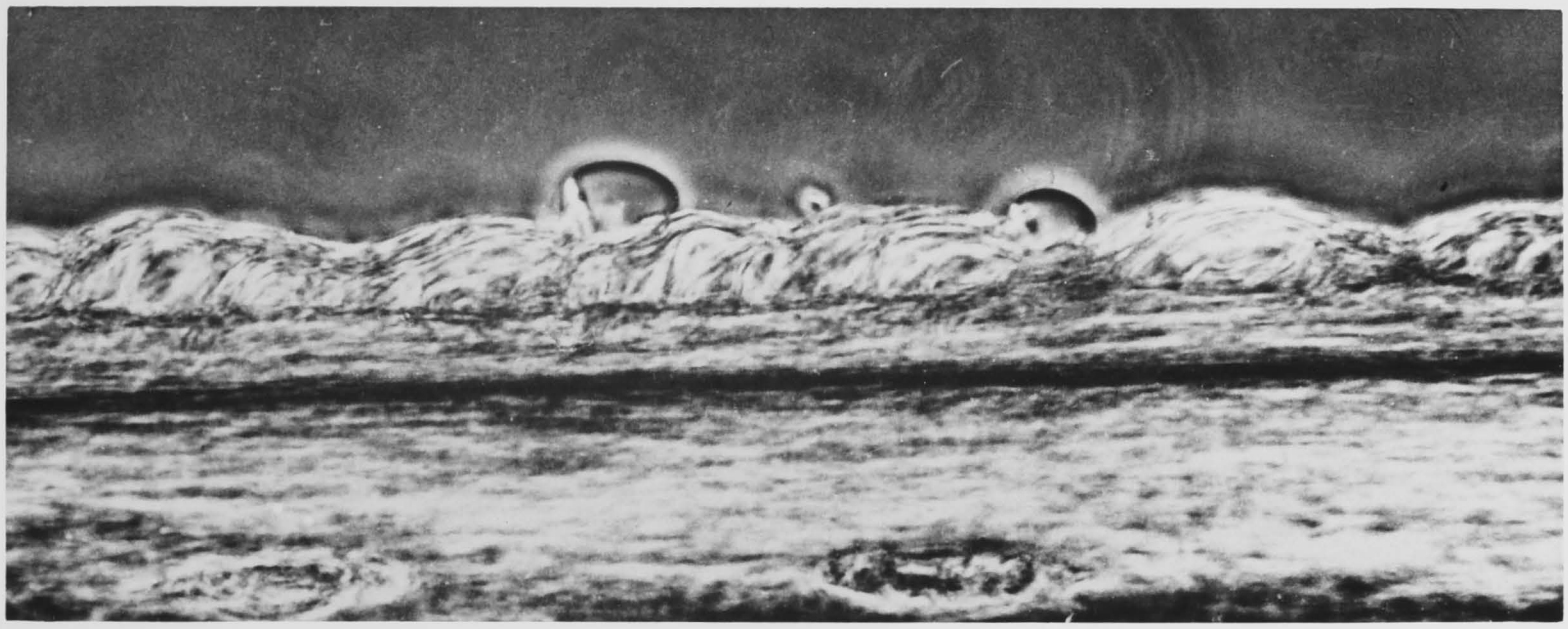


Figure 2.42. Effect of Chlorine Water on Human Hair.

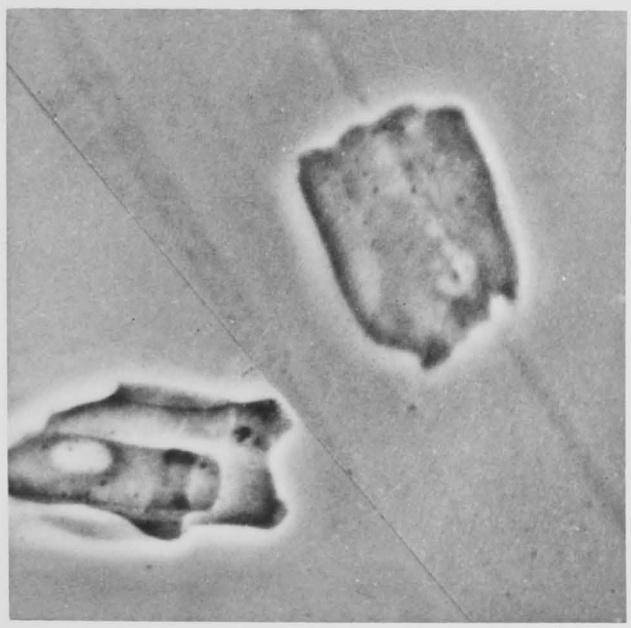


Figure 2.43. Cuticle Cells Isolated from Human Hair by HCOOH-treatment.

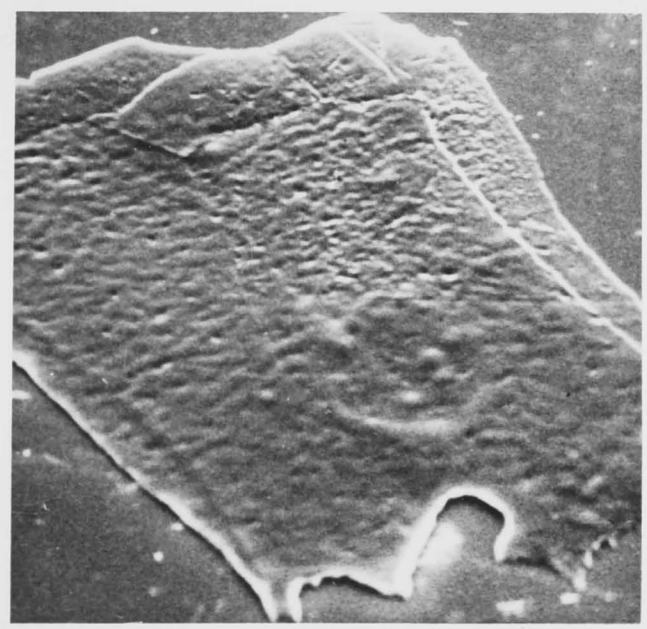


Figure 2.44. 'Stereoscan' of Cuticle Cells Isolated from Human Hair by HCOOH-treatment.

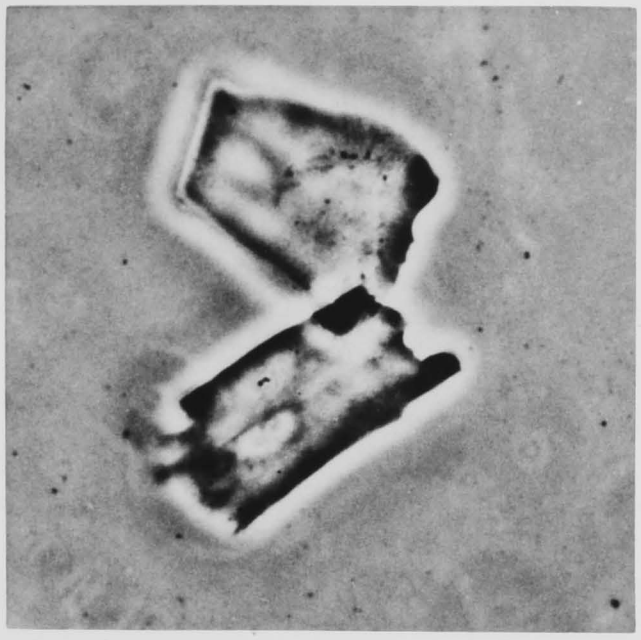


Figure 2.45. 20μ

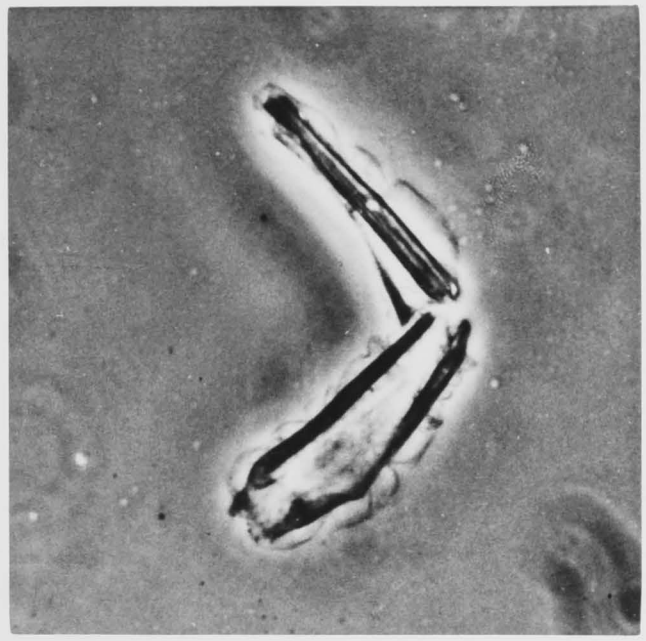
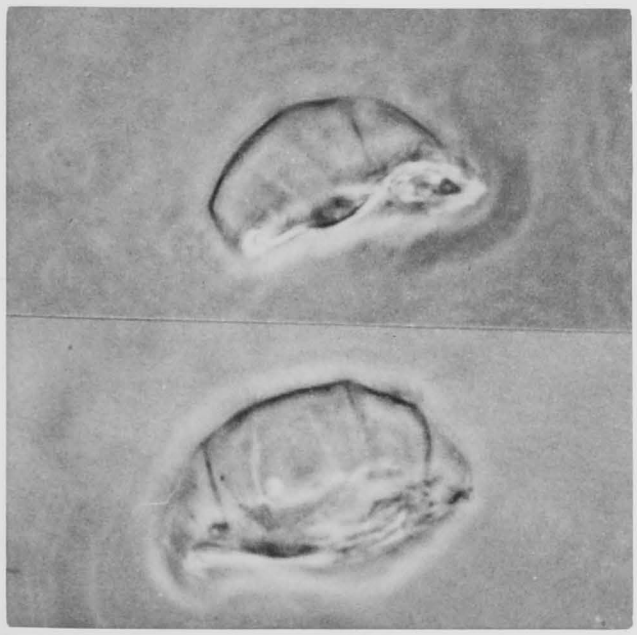


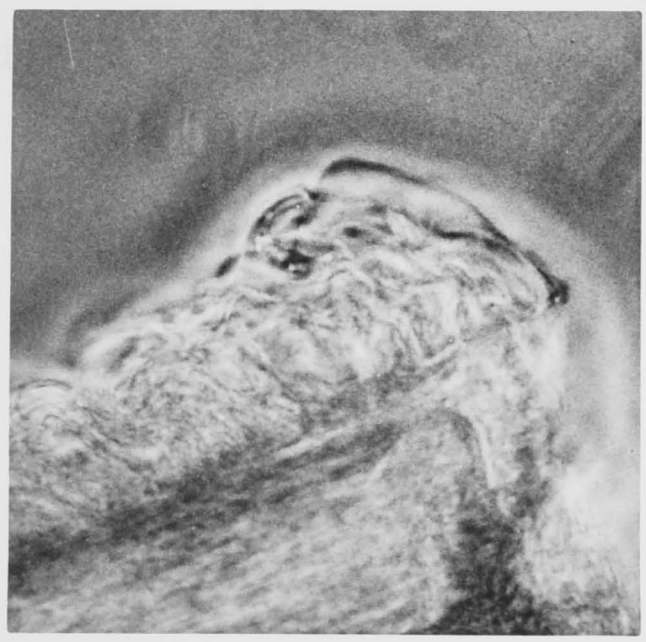
Figure 2.46. 20μ

Cuticle Cells from Human Hair, before and after Treatment with Chlorine Water.



20μ

Figure 2.47. Effect of Chlorine Water on Cuticle Cells Isolated from Human Hair.



20μ

Figure 2.48. Effect of Chlorine Water near the Cut End of a Human Hair.

The next step was to study dimensions and sac formation on isolated cuticle cells. Figures 2.43 and 2.44 show several such cells, the average dimensions of which were 30μ wide x 40μ long.

When chlorine water was added to a preparation of cuticle cells from human hair, almost every cell gave a positive Allworden reaction covering the whole scale, but again on one side only. The same effect was obtained with bromine water. Figures 2.46 and 2.47 are examples of such sac formation, while figures 2.45 and 2.46 show a typical 'before and after' effect of chlorine water. Note that although the scales in figure 2.45 were not edge-on to the camera, sac formation can be readily identified.

The formation of sacs covering one entire surface of isolated scales, when considered in relation to the occurrence of only a few small sacs on whole fibres, is further proof that epicuticle is associated with the individual cuticle cells and hence cannot be regarded as a continuous external membrane. Obviously only one-sixth of a sac covering an isolated cuticle cell represents epicuticle which was originally on the external surface of the intact fibre!

It was also noted that each sac which did form on the intact fibre was associated with a scale flap on the side nearest the proximal or basal end of the fibre (see

figure 2.42). This indicates that epicuticle sacs are 'trying' to form on every scale but are prevented from doing so in most cases by the next overlapping scale. Apparently the osmotic forces involved are not strong enough to induce sac formation over the limited exposed area of each cuticle cell, yet the co-operative effect is sufficient for the multiple-layered cuticle to undergo the observed disturbance.

To check the above interpretation, hairs were cut into short lengths and the Allworden reaction studied in the region of the cut ends. In all cases, large sacs similar to that shown in figure 2.48 appeared on the root end of the intact hair, in contrast to the smaller sacs which appeared on the uncut part of the fibre. Because of the large diameter of human hair, figure 2.48 does not do justice to the result obtainable - again, direct observation while varying the focus of the microscope gives a clearer appreciation of the effect.

Figure 2.49 summarises the effect of chlorine water on human hair. (see page 65).

The dimensions of isolated cuticle cells can be used to estimate the arrangement of these cells in the fibre surface. Since the length of a scale is 40μ and the length lying in the fibre surface is 6μ, the cuticle layer must be 6-7 cells thick. This agrees well with the value implied by

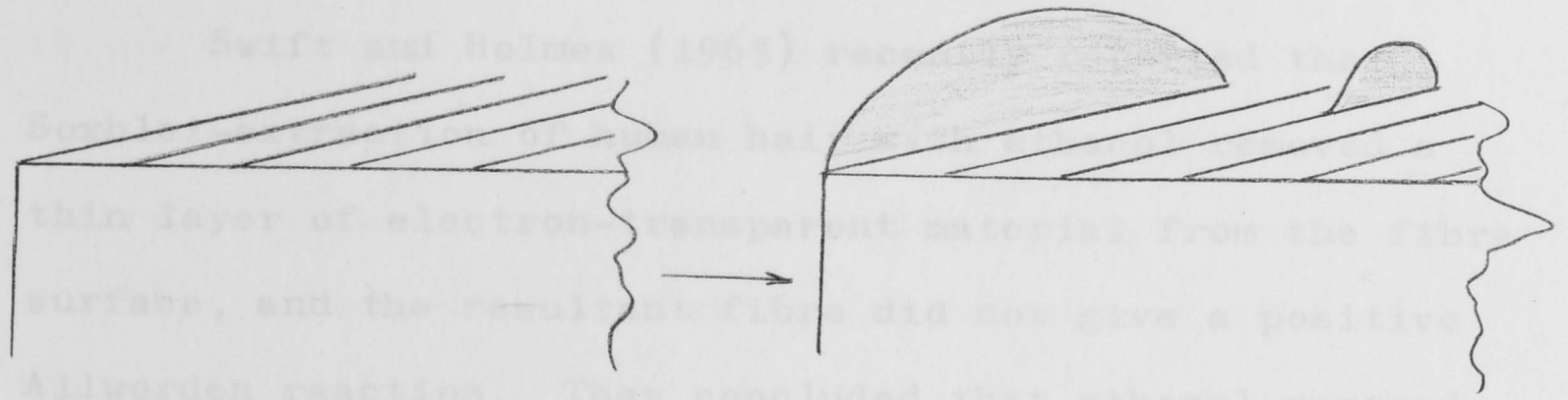


Figure 2.49. Schematic Representation of Allworden Sac Formation Near a Cut End of Human Hair.

Appleyard and Grevilles' (1950) observation that one-sixth of each scale is in the fibre surface. The width of a cell is 30μ and the fibre diameter is 125μ, so at least 16 cuticle cells are needed to encircle a fibre - considerably more than the estimate of 1-2 for Merino 64's fibres. A direct result of this is that formic acid treatment for 1 hour at 100°C produces large quantities of intact cuticle cells, with very little fragmentation.

The microscopical studies reported here gave no indication of false scale edges.

An interesting structural feature of the isolated cuticle cells was the 'holes' observed on all scales. Comparison of figures 2.43 (transmitted light) and 2.44 (incident electrons) shows that these are actually thin regions. Speculation on the origin and function of these

thin areas is outside the scope of the present work.

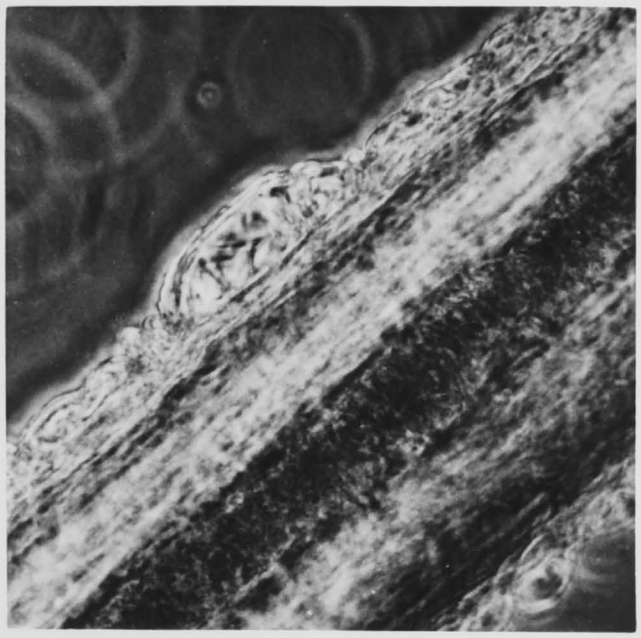
Swift and Holmes (1965) recently reported that Soxhlet-extraction of human hair with ethanol removed a thin layer of electron-transparent material from the fibre surface, and the resultant fibre did not give a positive Allworden reaction. They concluded that ethanol removed epicuticle, so considered this membrane to be composed of lipid material, and equated it with the lipid layer of the unit cell membrane. During the present work, Allworden sacs were observed to form on hair extracted with ethanol (and on hair treated for 1 hour at 100°C in formic acid!) just as readily as on untreated hair. This casts considerable doubt on the observations and interpretations of Swift and Holmes. King and Bradbury (1967) have firmly established that Merino 64's epicuticle is protein in nature.

The thickness and shape of the sacs formed on coarse animal hairs when immersed in bromine water are known to be different from those formed by chlorine water. Leveau et al. (1952) and Parisot and Leveau (1953) suggested that chlorine raised sacs enclosed by 'pure' epicuticle on fine wool, coarse wool, Siamese cat fur and human hair, while bromine lifted theentire cuticle layer on all except the fine wool. This general observation was endorsed by Schuringa et al. (1953). Fraser and Rogers (1955b) proposed that the 'epicuticle' raised on fine wool fibres

by bromine water was, in fact, epicuticle + the 'a' layer of the exocuticle. Thickness measurements on isolated membranes [King and Bradbury (1967)] support Fraser and Rogers' proposal, in that the bromine-membranes were approximately 5 times thicker than the chlorine-membranes of Merino 64's fibres.

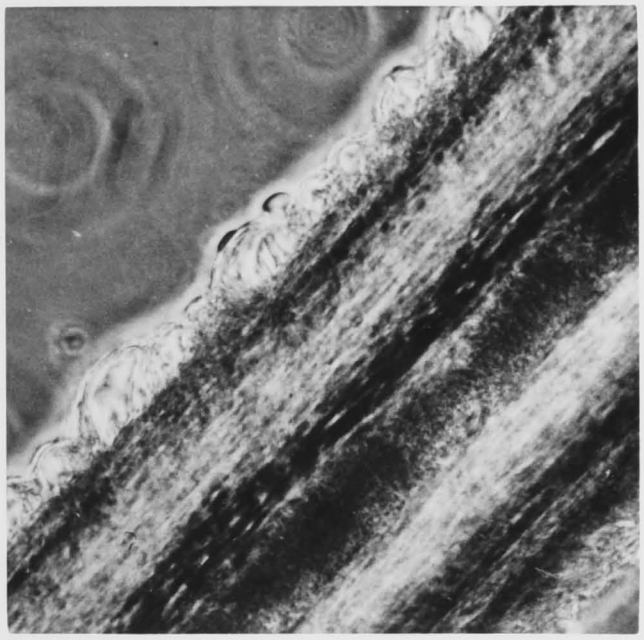
The effect of bromine water on untreated human hair is shown in figure 2.50. The surface swellings or 'bubbles' which form are obviously enclosed by a membrane much thicker than epicuticle. Scale edges can be seen on the surfaces of the sacs, confirming the claim of earlier workers that the whole cuticle is raised. This suggests that the cortex may be involved in the reaction and that the entire cuticle layer may be acting as a semi-permeable membrane [Leveau et al. (1952); Fraser and Rogers (1955b)]. In the words of Fraser and Rogers - "proteins released from the cortex by the action of bromine are unable to penetrate the cuticle, which therefore acts as a semi-permeable membrane. When sufficient osmotic pressure has developed, the cuticle detaches itself from the cortex and a cuticle-limited bubble is formed".

These authors also predicted that, with different fibres, the variable bromine reaction is due to variable scale overlap and to the nature of the cementing material between the scales.



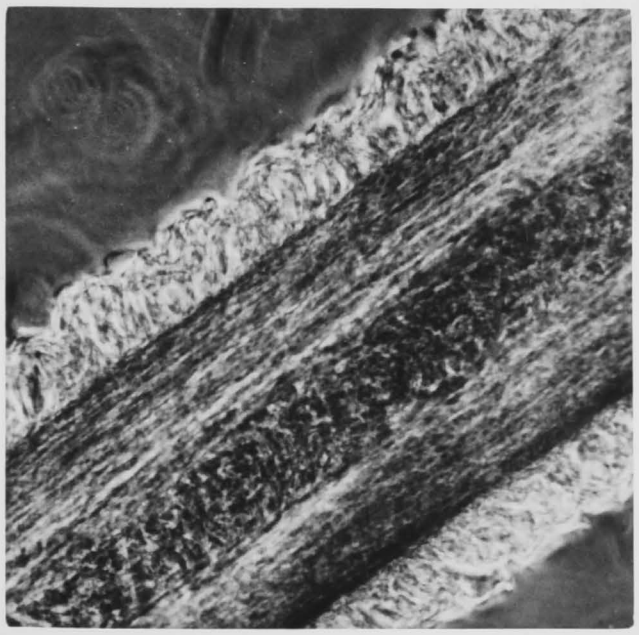
40μ

Figure 2.50. Effect of Bromine Water on Human Hair.



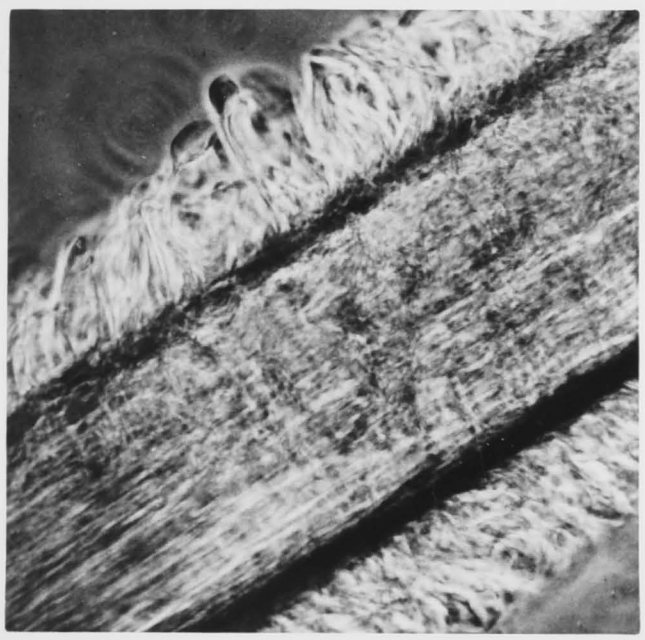
40μ

Figure 2.51. Effect of Adding Chlorine Water to Human Hair Immersed in Bromine Water.



40μ

Figure 2.52. Effect of Bromine Water on Human Hair Pretreated with Formic Acid.



40μ

Figure 2.53. Effect of Adding Chlorine Water to HCOOH-treated Human Hair Immersed in Bromine Water.

Human hair was used in the present work as a model substrate for studying the Herbig bromine-water reaction on fibres possessing a multiple-layered cuticle. Since chlorine water raises 'pure' epicuticle and bromine water raises the entire cuticle, there existed the possibility that addition of chlorine to bromine-treated fibres would raise epicuticle sacs on the cuticle sacs! This was found to be the case (figure 2.51), indicating a fundamental difference between the Allworden and Herbig reactions on human hair. The same result was obtained when the order of addition of the two halogens was reversed. This effect is surprising since no difference was observed between the reactions of bromine and chlorine on isolated scales - under phase contrast, the 140Å-thick membrane raised by bromine on Merino fibres had the same optical properties as the 30Å-thick membrane raised by chlorine. Furthermore, when the combined chlorine + bromine treatment was applied to Merino 64's fibres, formation of a second layer of sacs was not observed.

Fraser and Rogers (1955b) indicated that the intercellular cement may be partly responsible for the cuticle-limited swellings produced by bromine water. Separation of intact cuticle cells by treatment with formic acid depends on removal or disruption of this intercellular cement [Bradbury et al. (1965a, 1966, 1967)], so an obvious experiment was to add bromine water to human hair

which had been pretreated with formic acid. The result is shown in figure 2.52. No large cuticle-limited sacs were observed, but an occasional epicuticle-like sac appeared. Surprisingly, subsequent treatment with chlorine water again gave an additional effect (figure 2.53). The latter observation suggests that there are two differences between the Allworden and Herbig reactions on human hair - (i) the intercellular cement is somehow involved in the Herbig reaction, and (ii) the mode of action of chlorine and bromine <u>within</u> the individual cuticle cells is also different.

Although the above results show that the intercuticular cement is involved in the Herbig reaction on untreated hair, no information was obtained regarding the nature or site of attack by bromine (on the cortex?). The difference in length or area of intercuticular cement for human hair (five-sixths of each cuticle cell 'cemented') and Merino 64's (one-sixth of each cell 'cemented') probably accounts for the lack of whole-cuticle sacs when bromine water is applied to Merino fibres.

The suggested difference in mode of action <u>within</u> the cuticle cells may result from the difference in reactivity of chlorine and bromine towards keratin. It will be shown in section 2[E](iii) that Allworden sacs form at a much faster rate than Herbig sacs despite the fact that saturated bromine water is approximately 7 times the

concentration of saturated chlorine water. The difference in thickness of Herbig membranes also indicates a difference in site of reaction within the cuticle cells.

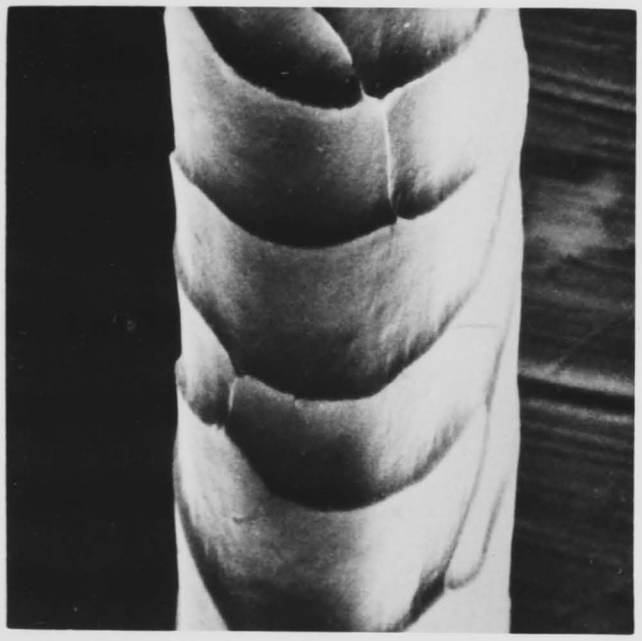
The present demonstration of involvement of intercellular cement in bromine-induced sacs under the cuticle layer, and of the effect of bromine in raising thin membranes when the available surface is large enough or free of mechanical constrictions, (e.g. sac formation on isolated scales) further invalidates the conclusions of Mercer and Golden (1953a,b). These authors compared their peracetic acid/sodium bicarbonate swellings to bromine-induced sacs and insisted (1953b) that "in all cases the (continuous)membranes may be described as epicuticle thickened by more or less of the adhering protein of the cuticular cells".

So again, here is an experimental result which should not be quoted as proof of continuous epicuticle.

(d) Kangaroo fur:

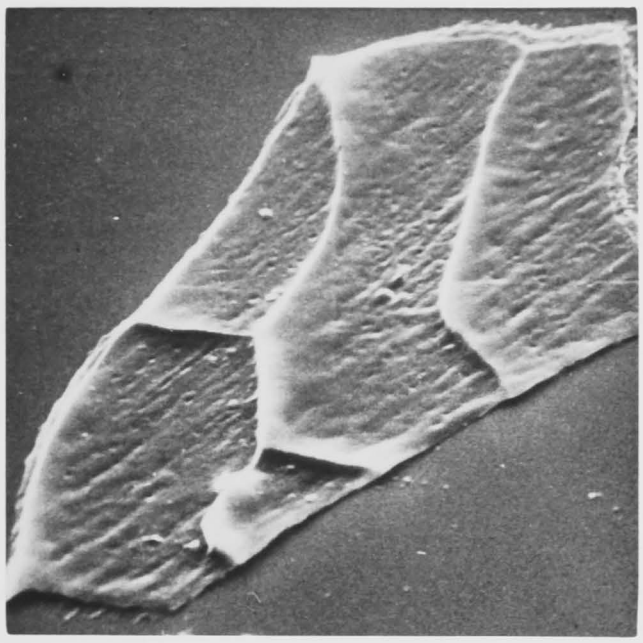
Scanning electron microscopy (figure 2.54) indicates that kangaroo fur closely resembles Merino fibres in diameter and surface structure, despite the occurrence in the former of a central core of medullary cells which form a large proportion of the fibre [Bradbury and O'Shea (1969); Bradbury et al. (1969)].

The Allworden sac pattern also appeared, at first



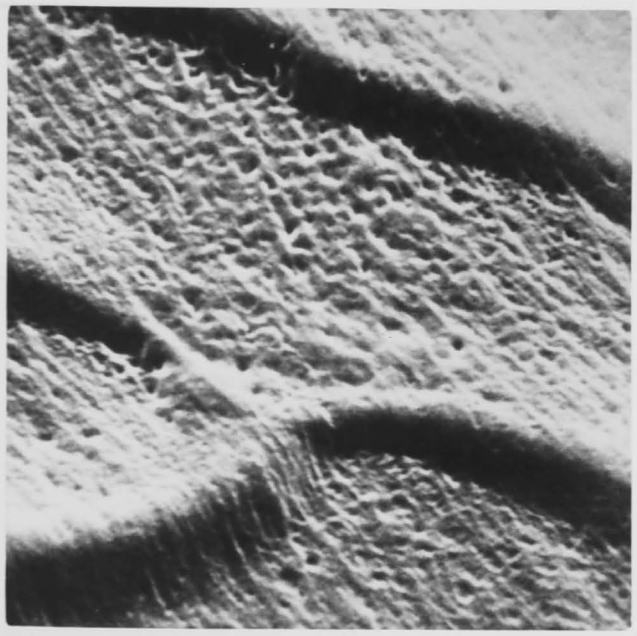
7μ

Figure 2.54. 'Stereoscan' of Kangaroo Fur Fibre.



7μ

Figure 2.55. 'Stereoscan' of Kangaroo Fur Cuticle Cell, Isolated by HCOOH-treatment.



2μ

Figure 2.56. 'Stereoscan' of Kangaroo Fur Cuticle Cell, Isolated by HCOOH-treatment.

20μ

Figure 2.57. Kangaroo Fur Cuticle Cells, Isolated by HCOOH-treatment.

sight, to be similar to that for Merino wool, but closer examination revealed significant differences in arrangement and structure of the scale layers. Figures 2.55 - 2.57 depict various aspects of isolated cuticle cells. Note the sharp 'scale edge' or rather, scale ridge in figure 2.55 and the smoother appearance when a similar fragment of cuticle material is viewed from the underside (figure 2.56). This suggests the frequent occurrence of false scale edges on kangaroo fibres. Further, examination of a large number of isolated scale fragments under the scanning electron microscope (e.g. figure 2.55) and the optical microscope (e.g. figure 2.57) revealed that all fragments were very similar in shape and approximate dimensions (30-50μ long x 20μ wide).

Allworden sac formation verified that such fragments represented whole cuticle cells - almost every fragment produced sacs, each of which covered one face of the cell (figure 2.58). This shows that, although the intact cuticle sheet (figure 2.59) has a similar surface pattern to Merino 64's, the arrangement of the individual cuticle cells cannot be the same. The average width of 20μ for kangaroo fur scales means that approximately twice as many cuticle cells occur around the circumference of this fibre compared with Merino fibre. This was substantiated by applying the Allworden reaction to kangaroo fibre cross-

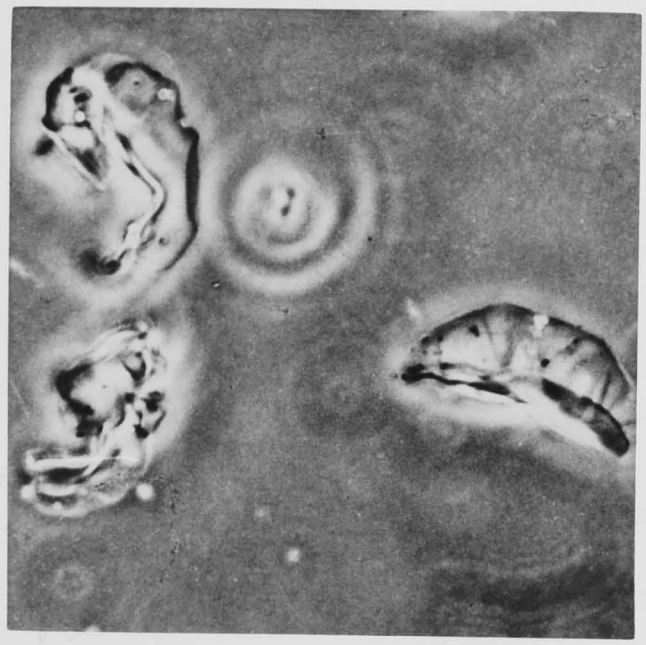


Figure 2.58. Effect of Chlorine Water on Isolated Kangaroo Fur Cuticle Cells.

Figure 2.59. Sheet of Kangaroo Fur Cuticle, Isolated by Two-stage HCl-HCOOH Treatment.

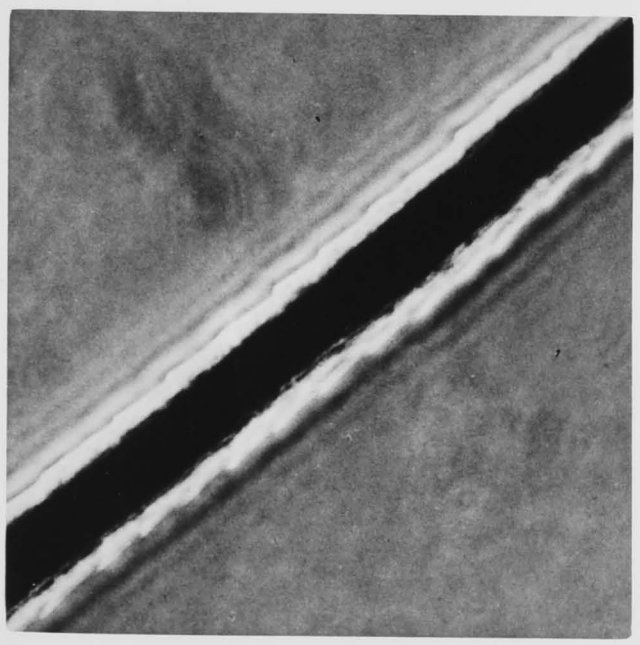


Figure 2.60. Kangaroo Fibre Mounted in Water.

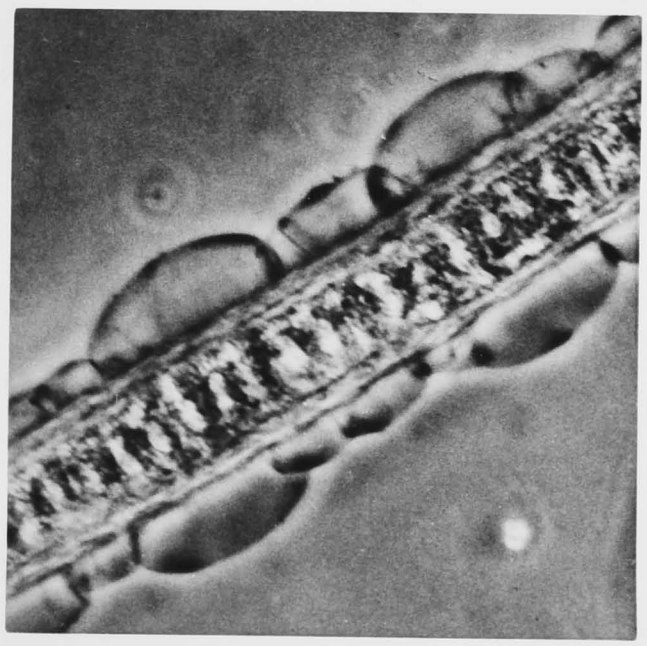


Figure 2.61. Effect of Chlorine Water on a Kangaroo Fibre.

sections, when as many as 6 small sacs were seen to form around the perimeter. Cross-sections greater than 50μ in thickness were needed to obtain full sac development around the fibre circumference, so 'publishable' photomicrographs could not be obtained because of focussing difficulties.

The distance between scale ridges (figure 2.60) was found to be 10-15μ and the longitudinal dimension of Allworden sacs (figure 2.61) was 20-45μ, showing that each cuticle cell has 2-3 false scale edges, compared with approximately one false scale edge on every second Merino cuticle cell. The significance of this observation will be considered later.

A consequence of this difference in arrangement of cuticle cells is that kangaroo fur is readily de-scaled by hot formic acid treatment, while smaller quantities of cuticle material containinga much smaller proportion of intact cells are released by similar treatments on Merino 64's fibres.

One point regarding interpretation of sac formation on isolated scales should be mentioned here - the assumption has been made that the longest dimension of the scale is always measured, due to preferential alignment in the restricted space between the microscope slide and cover slip. This measurement would be the transverse dimension for most Merino cuticle cells and the longitudinal dimension

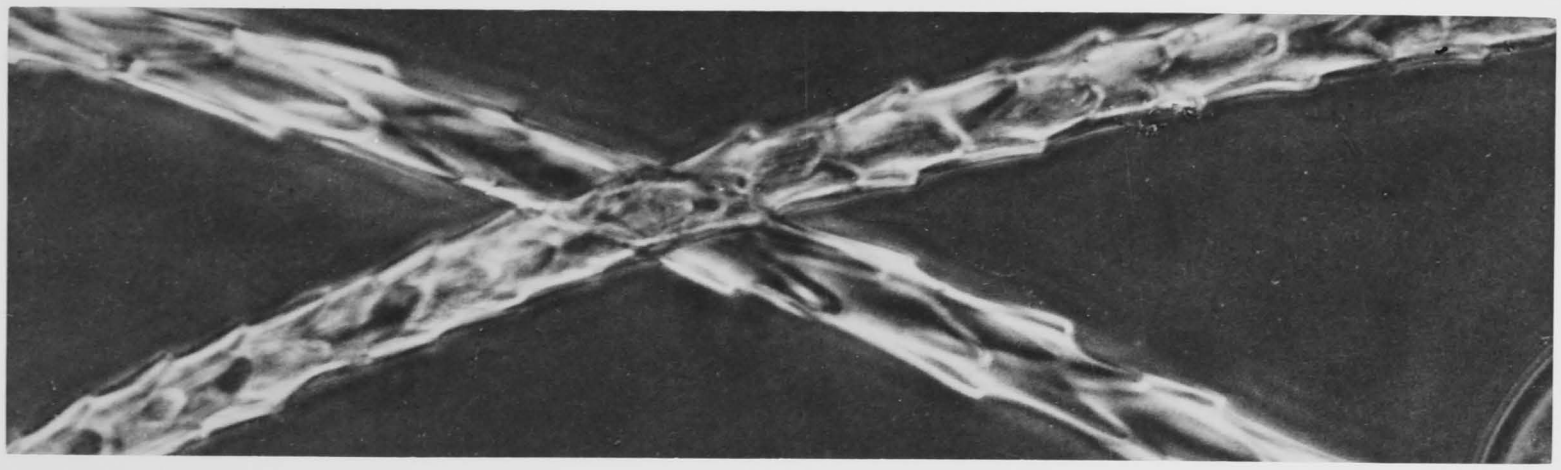
for scales from human hair and kangaroo fur. Microscopical observations without the addition of a cover slip, and other considerations in the body of the text, support this assumption.

(e) Seal fur:

Seal fur fibres are approximately 15μ in diameter, so are finer than Merino 64's fibres. Figure 2.62 and 2.63 show typical seal fur fibres before and after addition of chlorine water. Note the striking surface profile and the association of an Allworden sac with each 'scale tip'. The sacs formed very slowly, 30-60 minutes being required for full sac coverage compared with 15 seconds for Merino fibres. The photomicrograph shown in figure 2.63 was taken 15 minutes after addition of chlorine to illustrate the mode of sac formation.

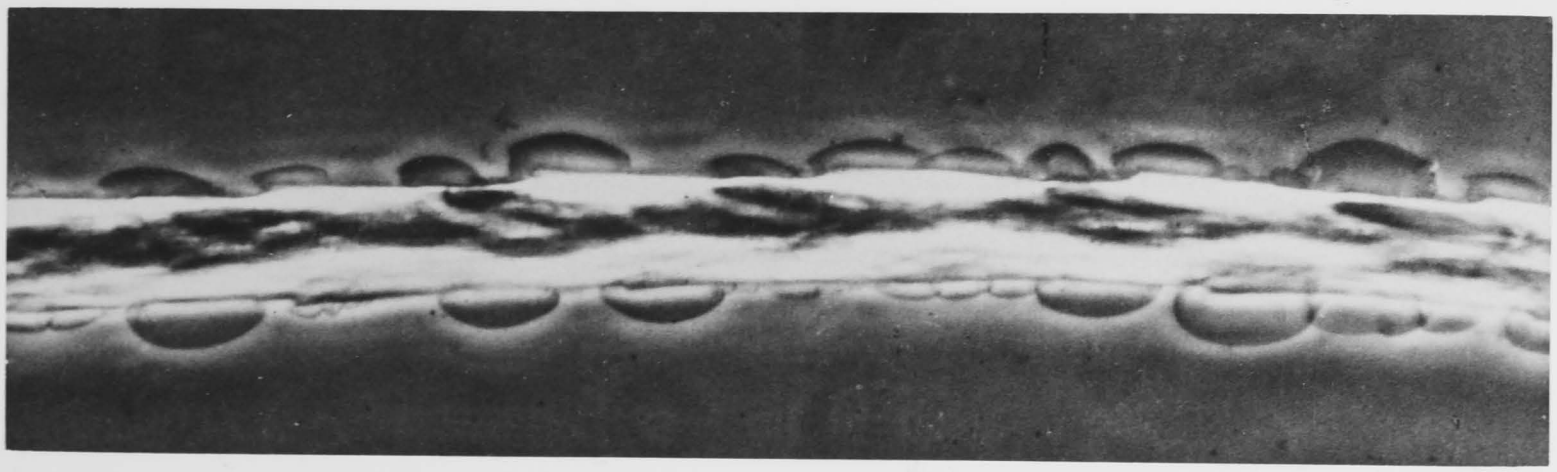
Further study of the relation between epicuticle and scale structure was not possible. The fibres refused to break down under the conditions employed for other keratin fibres. Treatment with 0.01N hydrochloric acid at 100°C for 20 hours followed by boiling in 98-100% formic acid for 20 hours and subsequent severe agitation resulted in separation of some scale (?) material, but immersion of these irregular-shaped fragments with chlorine water or bromine water did not induce sac formation.

The scanning electron micrographs in figure 2.64



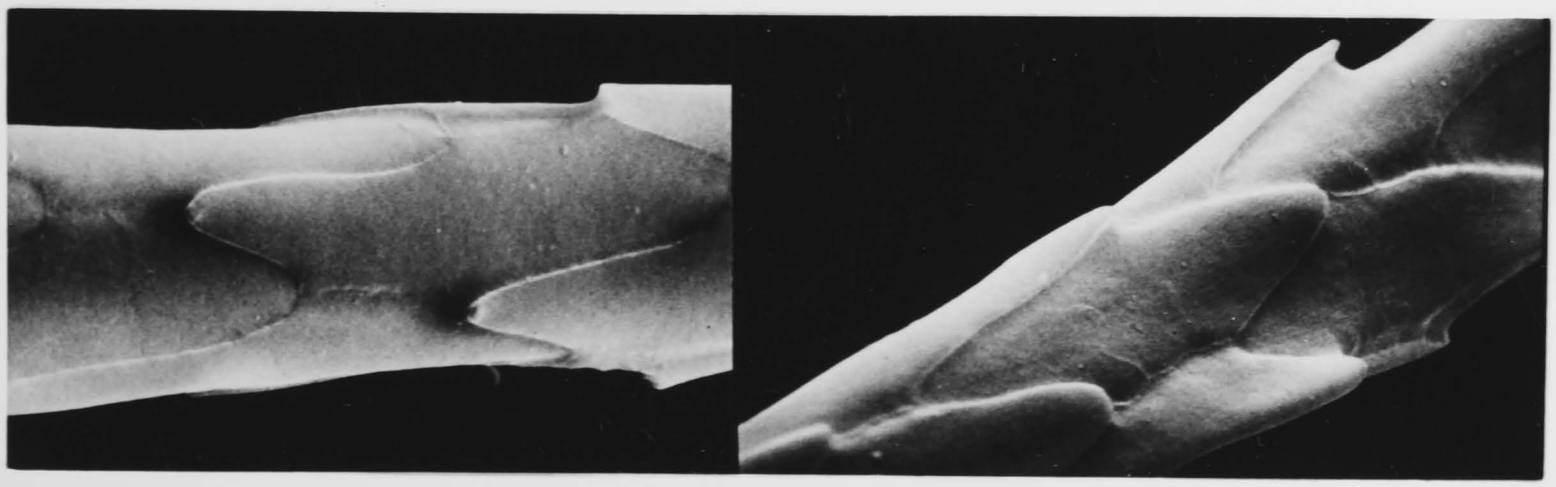
20μ

Figure 2.62. Seal Fur Fibre, Mounted in Water.



20μ

Figure 2.63. Effect of Chlorine Water on Seal Fur Fibre.



5μ

Figure 2.64. 'Stereoscans' of Seal Fur Fibre.

clearly illustrate the scale pattern. The individual scales (if they exist!) are apparently strongly bonded to each other and/or to the body of the fibre. No surface cracks or crevices can be seen, although the fine longitudinal striations (figure 2.64) could perhaps represent the junction between cuticle cells.

The extreme resistance to disruption exhibited by seal fur may be a reflection of the value placed on this fibre by the fur industry - seal fur enjoys a status somewhat above that of mink [J. Menkart, private communication to J. H. Bradbury].

(f) Seal guard hair:

Seal guard hairs are much more variable in diameter and scale structure than seal fur. Measurements from photomicrographs gave fibre thickness values ranging from 40μ to more than 200μ. Of greater interest, however, is that the scale pattern shows considerable variation along the length of the individual fibres. The occurrence of such variable cuticle cell arrangement in some types of keratin fibres was noted by Hardy and Plitt (1940), Lyne and McMahon (1951) and Wildman (1955).

Figures 2.65 - 2.67 illustrate the three distinct types of surface structure observed on seal hair. Following the nomenclature of Lyne and McMahon (1951), these three regions will be termed basal (near root end of fibre),

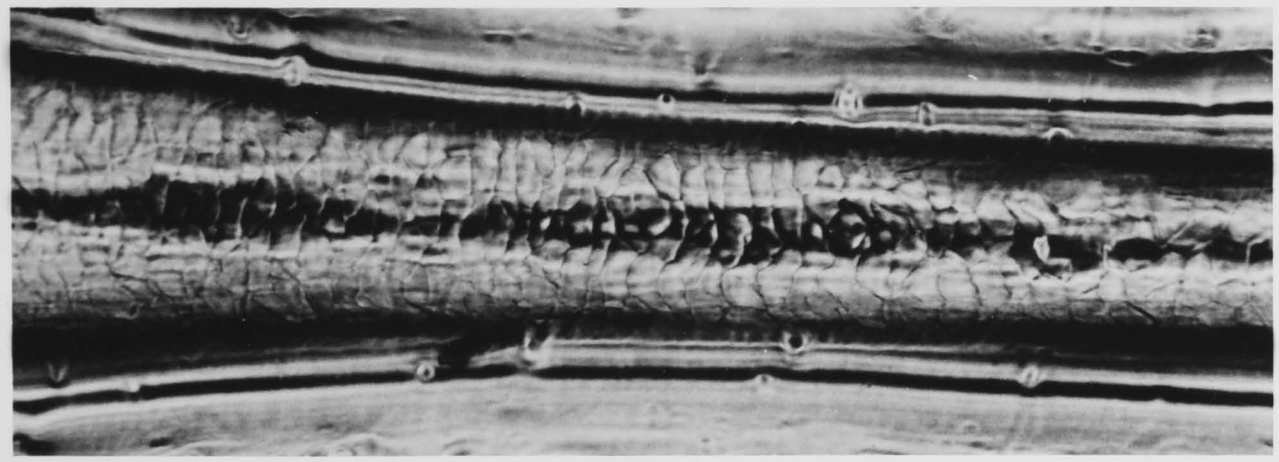


Figure 2.65. Seal Hair (Basal) -'Sellotape' Mount.

40μ

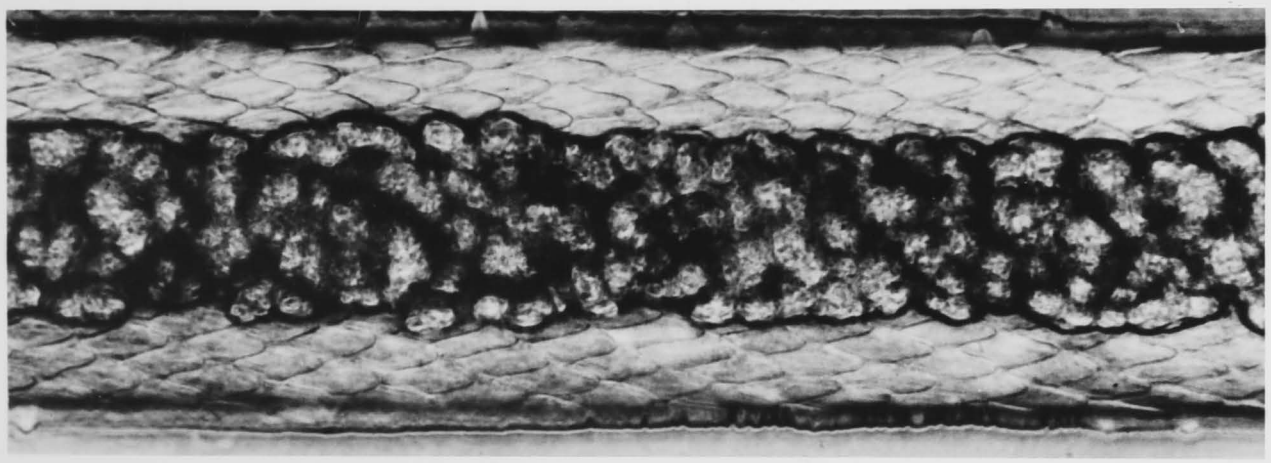


Figure 2.66. Seal Hair (Mid-shaft) -'Sellotape' Mount.

40μ

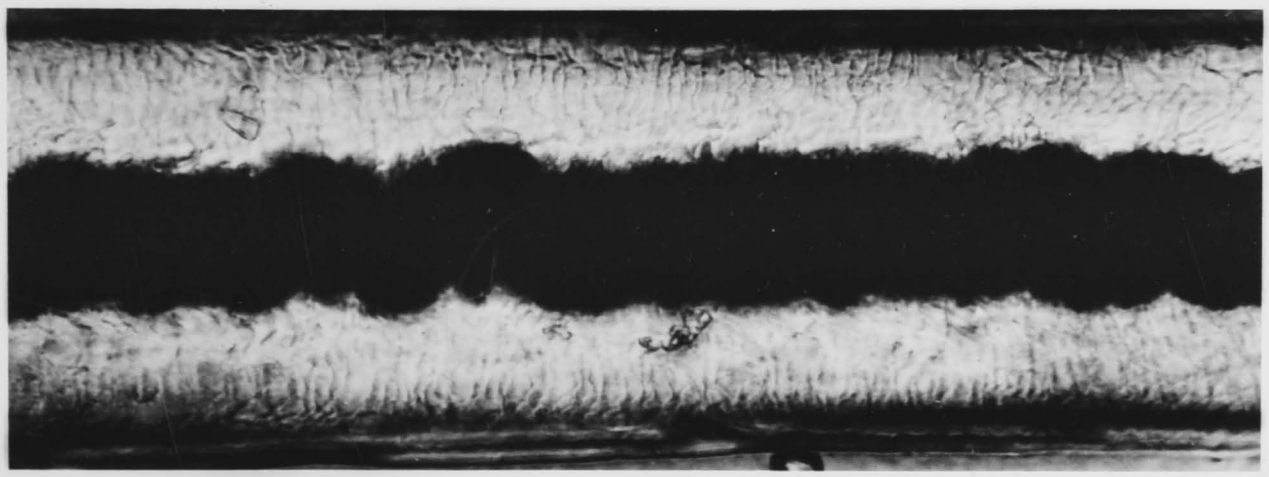


Figure 2.67. Seal Hair (Distal) -'Sellotape' Mount.

40μ

mid-shaft, and distal (near tip of fibre). The basal cuticle cells are arranged similarly to those on the coarser wool fibres [see, e.g. Wildman (1955); Kassenbeck (1959)]; the mid-shaft region exhibits a 'diamond'-type pattern somewhat similar to that on seal fur; and the distal end of the fibre has a multiple-cuticle structure closely resembling that of human hair.

The effects of chlorine water on these three regions are illustrated in figures 2.68 - 2.70. The sac patterns conform to those indicated by the scale patterns of untreated fibres. The basal region gives similar sac formation to Merino, Lincoln and kangaroo fibres; the distal ends give a reaction typical of human hair; and the mid-shaft region slowly develops sacs in a similar manner to seal fur.

Seal hair is not circular in cross-section over its whole length, since ribbon-like folds were often observed in the transition regions between the various types of scale pattern. Figure 2.71 shows Allworden sacs near such a fold.

Differences in shape of the isolated cuticle cells were also observed. Figures 2.72 and 2.73 show scales from the distal region, before and after addition of chlorine water, while figures 2.74 and 2.75 show similar preparations from the basal or root end. Note the resemblance between the latter amd kangaroo fur cuticle.

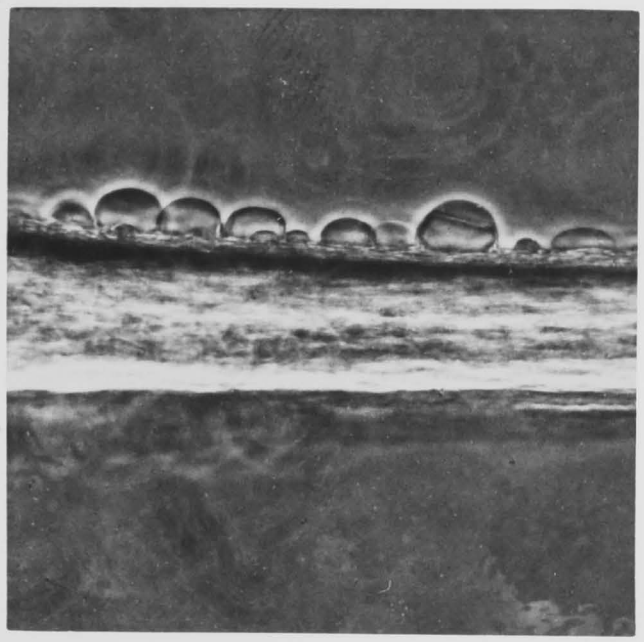


Figure 2.68. Effect of Chlorine Water on Seal Hair (Basal).

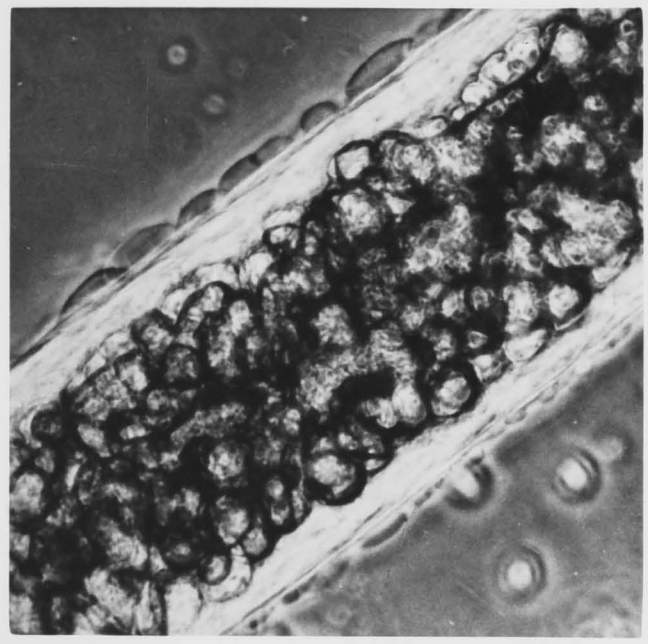


Figure 2.69. Effect of Chlorine Water on Seal Hair (Mid-shaft).

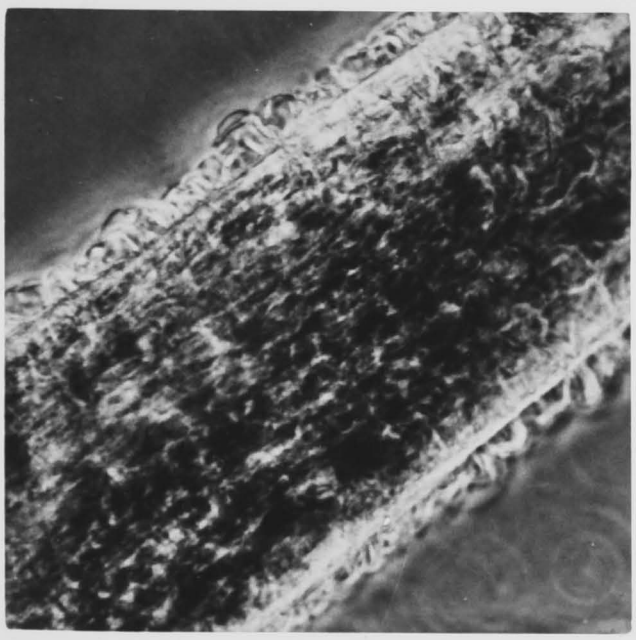


Figure 2.70. Effect of Chlorine Water on Seal Hair (Distal).

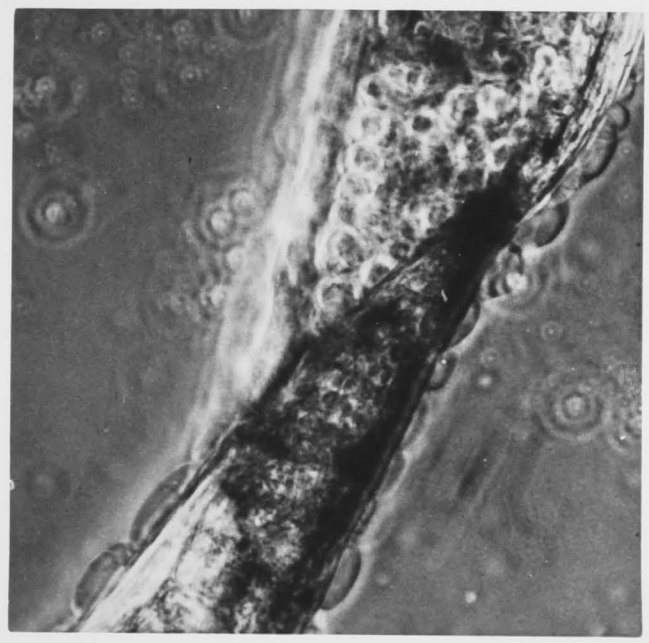


Figure 2.71. Effect of Chlorine Water near a 'Folded' Section of Seal Hair.

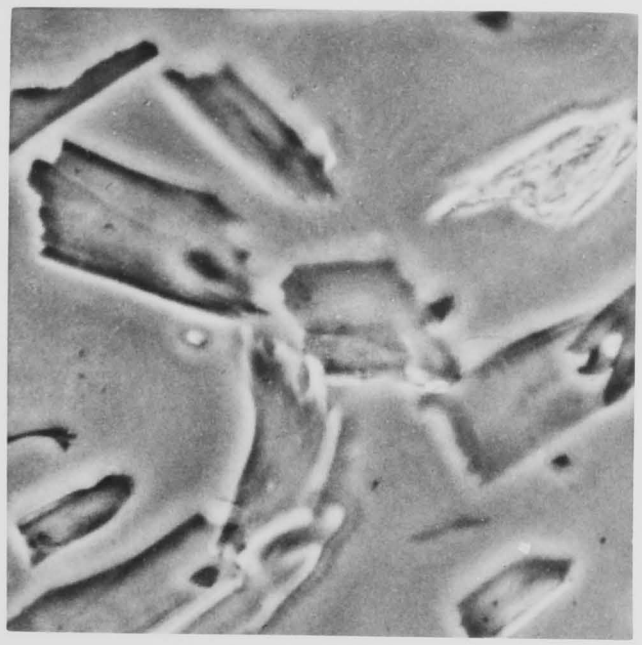


Figure 2.72. Seal Hair Cuticle Cells (Distal), Isolated by HCOOH-treatment.

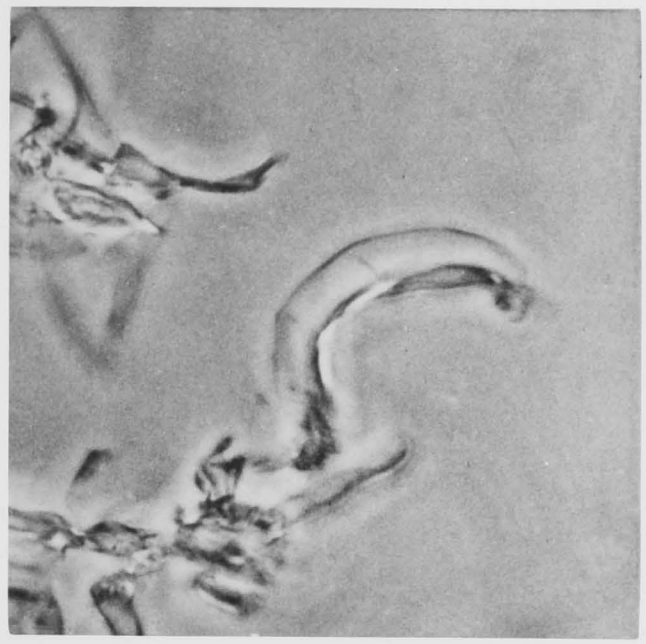


Figure 2.73. Effect of Chlorine Water on Seal Hair Cuticle Cells (Distal).

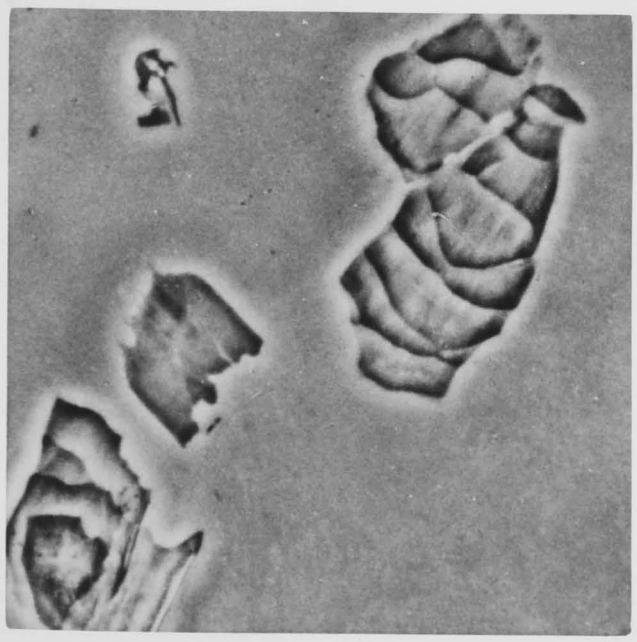
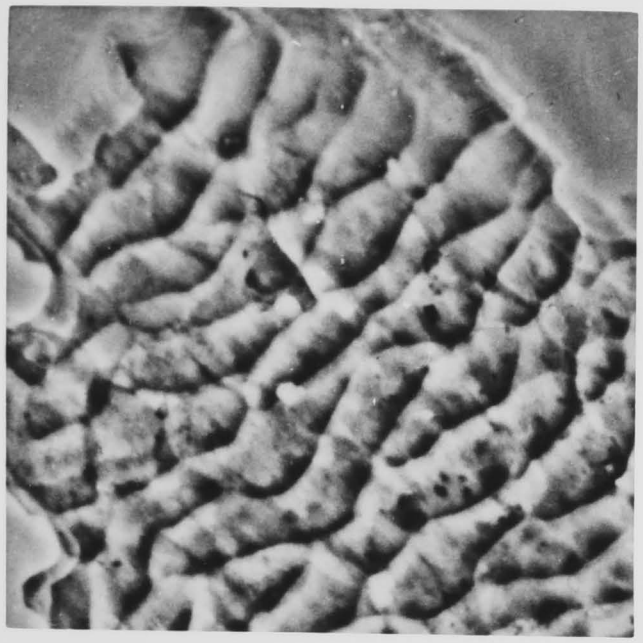


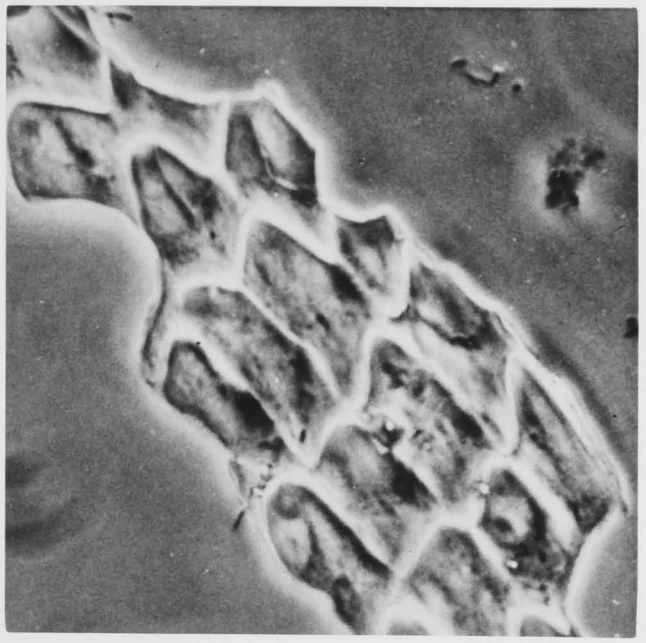
Figure 2.74. Seal Hair Cuticle Cells (Basal), Isolated by HCOOH-treatment.

Figure 2.75. Effect of Chlorine Water on Seal Hair Cuticle Cells (Basal).



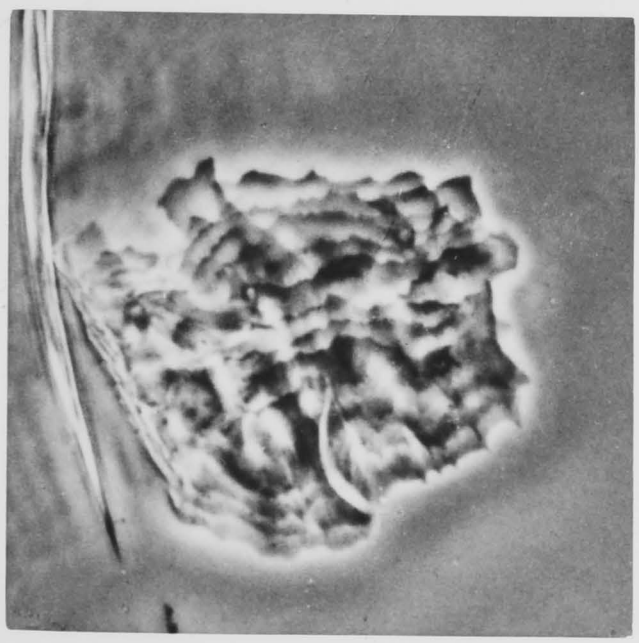
20μ

Figure 2.76. Cuticle Sheet from Seal Hair (Basal).



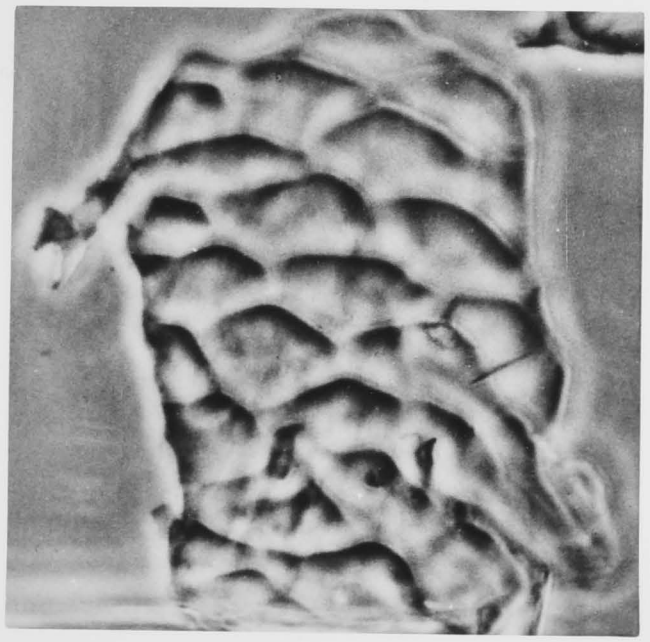
20μ

Figure 2.77. Cuticle Sheet from Seal Hair (Mid-shaft).



20μ

Figure 2.78. Cuticle Sheet from Seal Hair (Distal).



20μ

Figure 2.79. Cuticle Sheet from Seal Hair (Transition-region).

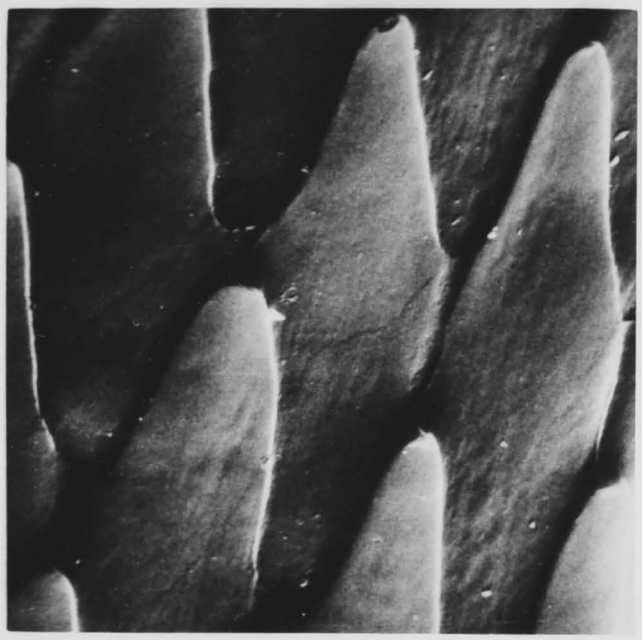


Figure 2.80. 6μ

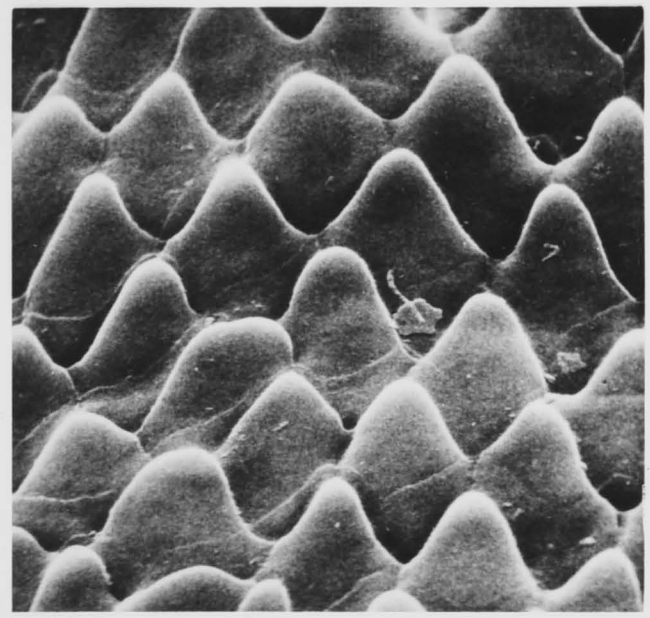


Figure 2.81. 10μ

Figures 2.80-2.83. 'Stereoscans' of Seal Hair, Showing Gradual Transition from Single-cuticle 'Diamond' Scale Pattern to Multiple-cuticle Pattern.

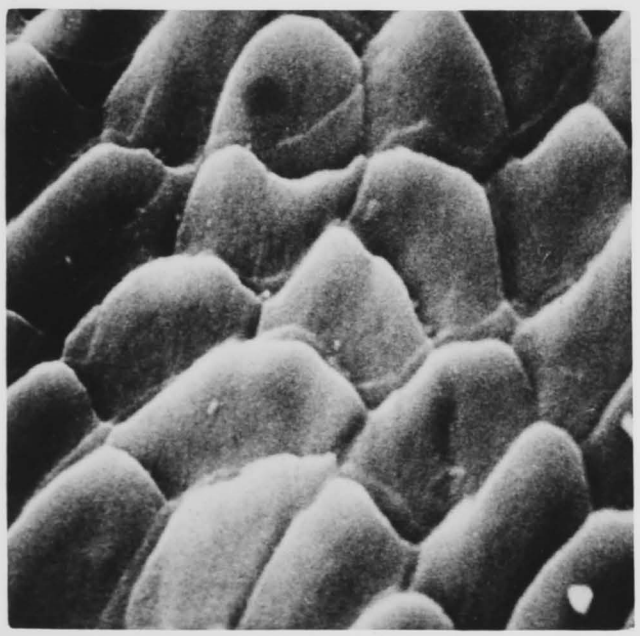


Figure 2.82. 10μ

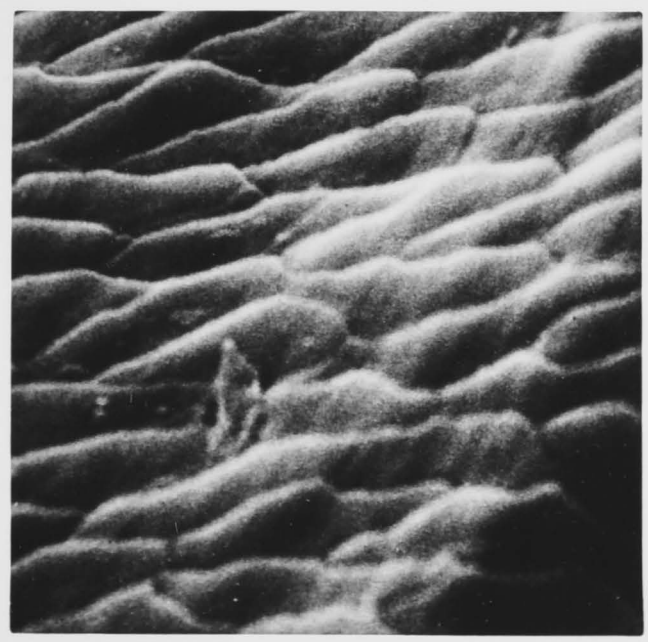


Figure 2.83. 10μ

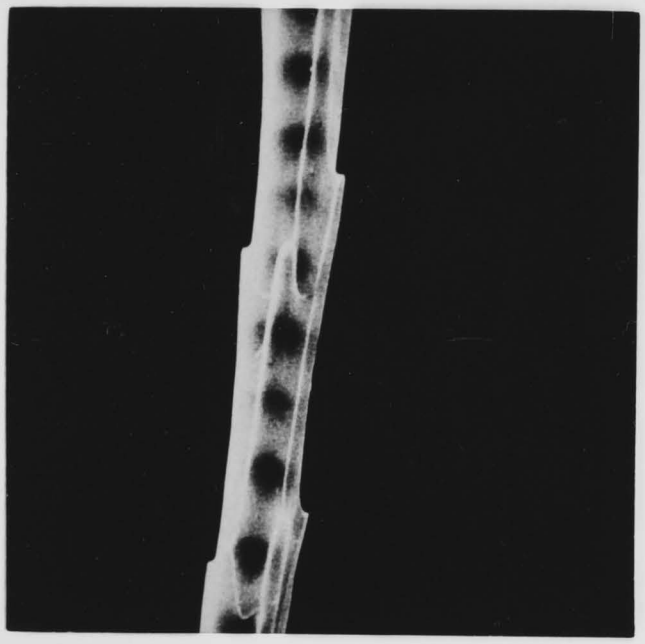
Single cuticle cells from the mid-shaft region could not be obtained, again showing the similarity between the surface structure of this region and that of seal fur fibres.

Photographs of cuticle sheets isolated by the two-stage HCl-HCOOH treatment, are presented in figures 2.76 - 2.79. A large number of different structures were observed, corresponding to various stages of the transitions from basal to mid-shaft patterns, or mid-shaft to distal patterns. One such preparation is shown in figure 2.79, while figures 2.80 - 2.83 are scanning electron micrographs showing the gradual change from mid-shaft structure to distal structure.

As far as the author is aware, biologists have not yet studied the factors involved in production of the changing scale patterns along the same fibre. Obviously some change is involved in biosynthesis of the fibre in the hair follicle - this aspect will be briefly discussed later in the present section.

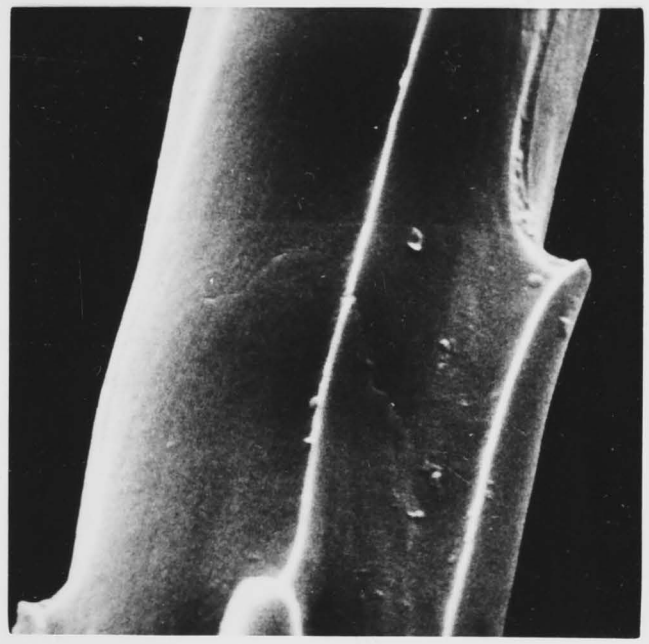
(g) Platypus fur:

Fibres of platypus fur are even finer than those of seal fur, ranging in thickness from 10μ to 15μ. Figures 2.84 and 2.85 indicate that the scale structure is similar to that of seal fur. The black spots along the centre of the fibre result from the presence of medullary cells, but it is surprising that they are visible under the scanning electron microscope, which supposedly 'sees' only surface



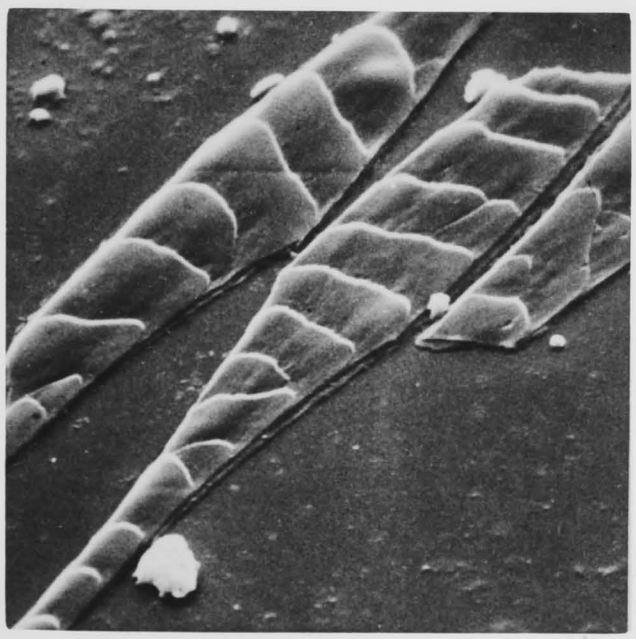
10μ

Figure 2.84. 'Stereoscan' of Platypus Fur.



2μ

Figure 2.85. 'Stereoscan' of Platypus Fur.



7μ

Figure 2.86. 'Stereoscan' of Platypus Fur Cuticle, Isolated by HCOOH-treatment.

20μ

Figure 2.87. Platypus Fur Cuticle, Isolated by HCOOH-treatment.

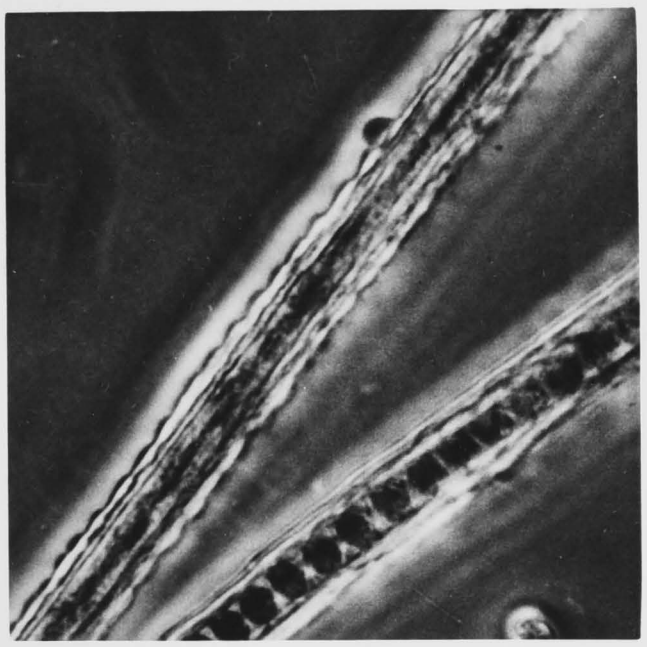
detail. This effect probably reflects the observation that platypus fur consists mainly of medulla and cuticle, with a thin layer of cortical cells sandwiched between [Bradbury et al. (1969)]. The limited penetration of electrons in the scanning electron microscope is apparently sufficient to delineate the central medullary cells.

Because of the similarity in surface structure of platypus fur and seal fur, separation of cuticle was not expected to occur. However, large quantities of material such as that shown in figures 2.86 and 2.87 readily separated during the standard HCOOH-treatment; furthermore, this material bore no resemblance to the surface structure observed on whole fibres. Microscopical studies on various fur components [Bradbury et al. (1969)] established that the material was not of cortical or medullary origin, yet the 'ladder-type' surface markings and length of the cells suggested that they did not originate from the cuticle.

Close studies of the Allworden reaction on whole fibres suggested a reason for this anomaly. Figures 2.88 and 2.89 show theeffect of chlorine water on the basal and distal regions, respectively, and indicate that two types of cuticle cell occur in platypus fur. This was further indicated from studies on the material produced by the two-stage HCl-HCOOH treatment - sheets of cuticle material were not produced, but a small proportion of fragments such as

20μ

Figure 2.88. Effect of Chlorine Water on Platypus Fur - Basal.



20μ

Figure 2.89. Effect of Chlorine Water on Platypus Fur - Distal.

20μ

Figure 2.90. Scale Fragments Isolated from Platypus Fur by Two-stage HCl-HCOOH Treatment.

20μ

Figure 2.91. Effect of Chlorine Water on a Cuticle Cell from the Distal End of a Platypus Fur Fibre.

those pictured in figure 2.90 were observed. This corresponds to the scale pattern near the base of the fibre. These fragments did not give a positive Allworden reaction, in contrast to the 'ladder-type' cuticle cells, which developed many small sacs (see figure 2.91) conforming to the numerous transverse ridges seen in figures 2.86 and 2.87.

The apparent anomaly arose because the scanning electron micrographs represented only the basal regions of the fibre, while the difference in resistance to breakdown in formic acid had resulted in separation of cuticle material exclusively from the distal end.

Final proof of the occurrence of the two types of surface structure was obtained by careful re-examination of untreated platypus fur under the optical microscope. When mounted in water the basal and mid-shaft regions of the fibre showed a scale profile equivalent to that seen in the scanning electron micrographs, with a gradual transition to an apparently featureless surface near the distal end. However, surface structure corresponding to that indicated by the majority of isolated scales, became visible when the fibre was mounted in 'Sellotape'. Figure 2.92 shows the base and tip ends of a single fibre, mounted in 'Sellotape'.

Despite the occurrence of many small sacs on the surface of intact fibres (figure 2.89) and on distal cuticle

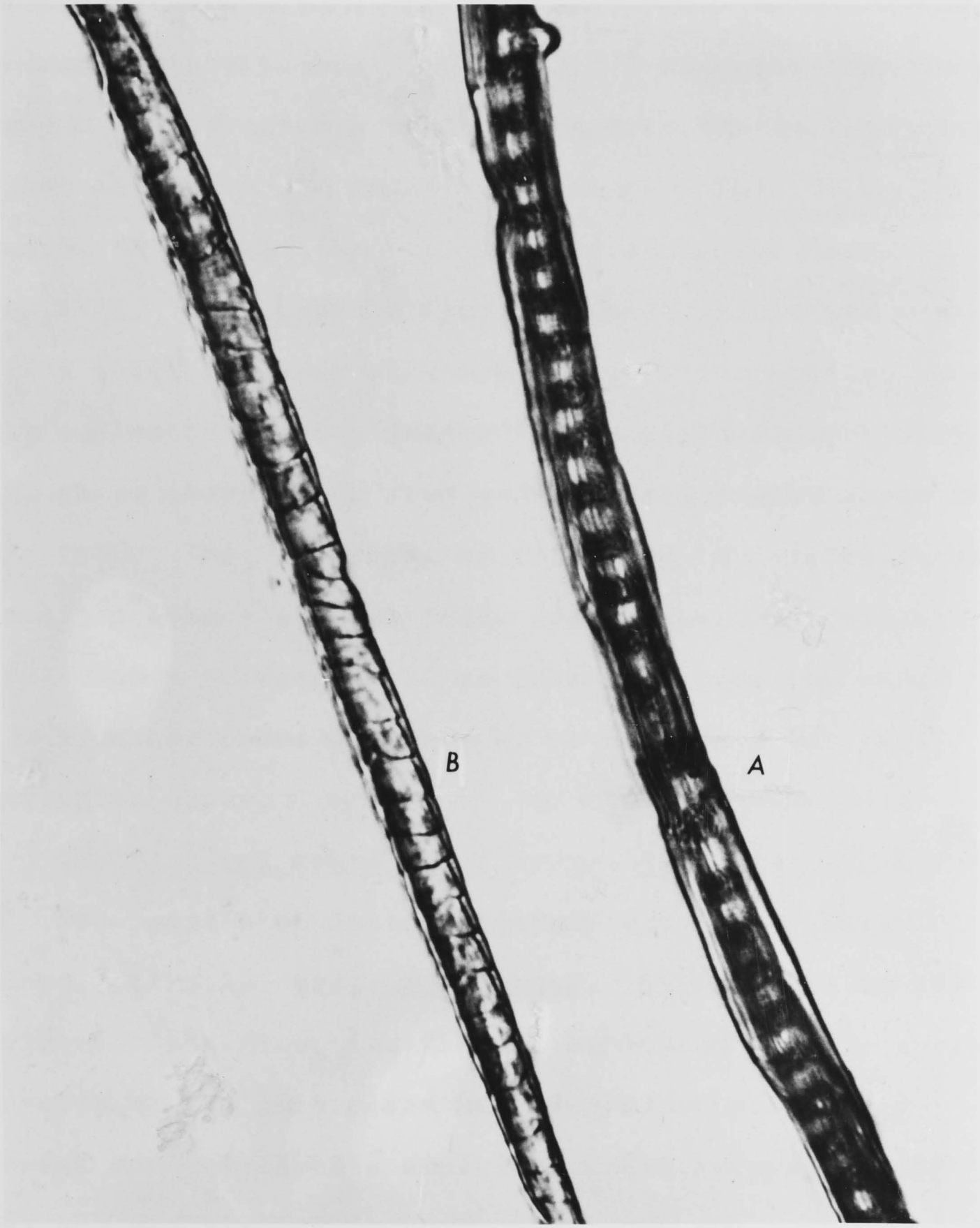


Figure 2.92. Single Platypus Fur Fibre, Mounted on 'Sellotape';
(A) Basal and (B) Distal.

fragments (figure 2.91), the similarity in size and shape of these fragments (figures 2.86 and 2.87) suggests that they represent single cuticle cells. The numerous surface ridges are then assumed to be false scale edges. This is an extension of the similar postulate advanced for kangaroo fur cuticle. Thus, when a fibre was left in contact with chlorine water for several hours, some of the smaller sacs slowly coalesced to form longer flat swellings, optically identical to those which form on the basal region shown in figure 2.88. The narrowness of fibre and cuticle cell, and the small distance between false scale edges, may explain the reluctance of epicuticle to form sacs over the whole cell - a larger osmotic force may be necessary for sac formation to occur.

(h) Platypus guard hair:

Two samples of coarse platypus guard hair were examined. Firstly, belly guard hair, which occurs in admixture with the finer fur fibres, exhibited similar surface structures to the seal guard hair described previously. The basal end possessed a wool-like scale structure, the distal end was similar to human hair, and the cuticle of the mid-shaft region resisted separation from the parent fibre as did that of seal hair. The mid-shaft scale pattern was somewhat different from that of seal hair, but had the general appearance of the basal end of platypus fur.

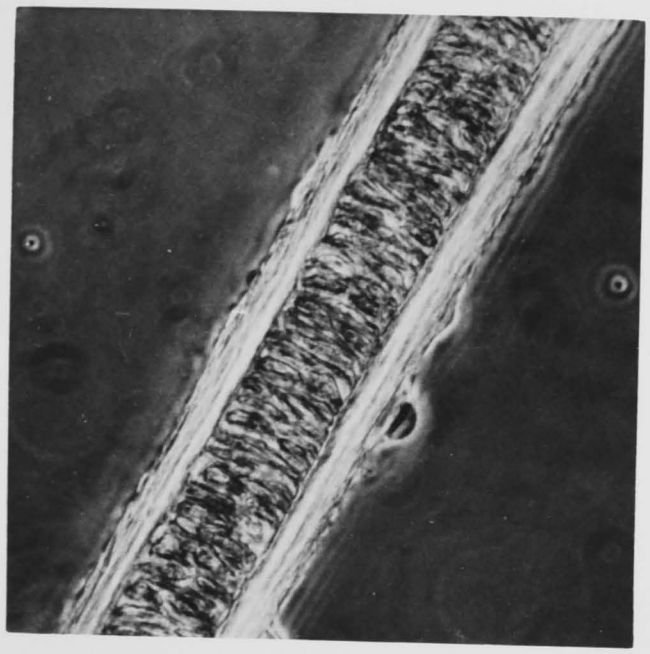
Figure 2.93 shows the (limited) Allworden sac pattern on this region. In all other respects the illustrations and discussion of seal guard hair apply equally well to platypus belly guard hair.

Figures 2.94 - 2.96 are scanning electron micrographs of the three types of scale structure found along a single fibre.

The second platypus guard hair sample was from the tail region. It contained no contaminating fur fubres. Unlike the belly guard hairs, variation in scale pattern along the individual fibres did not occur.

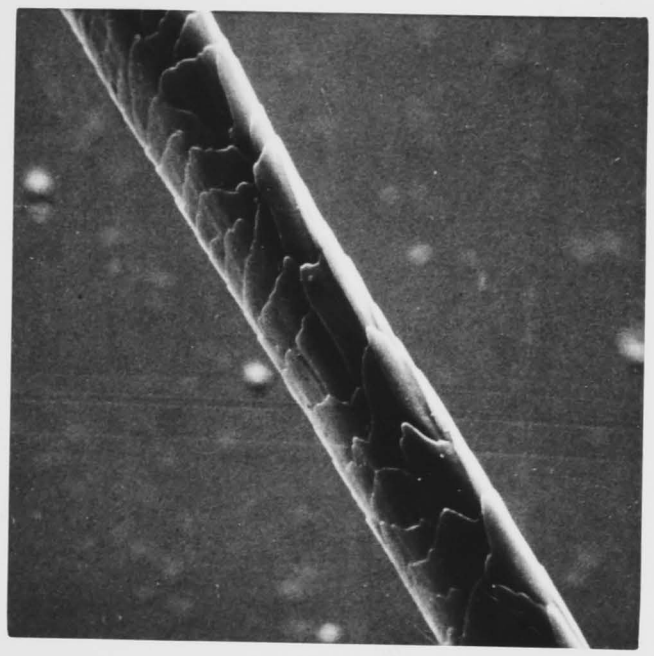
Micrographs of isolated scales (figure 2.97), a sheet of cuticle material (figure 2.98) and a 'Sellotape' mount of an intact fibre (figure 2.99) reveal that the shape and arrangement of the cuticle cells are similar to those of human hair. Allworden sac formations on intact fibres and on isolated cuticle cells were also very similar to those on human hair.

When chlorine water was applied to fibres partially descaled by the standard HCOOH-treatment, the effect shown in figure 2.100 was obtained. This provides further indications that (1) epicuticle covers the individual cuticle cells, and (2) Allworden sacs will form over one entire surface of the scale when mechanical constraints are removed.



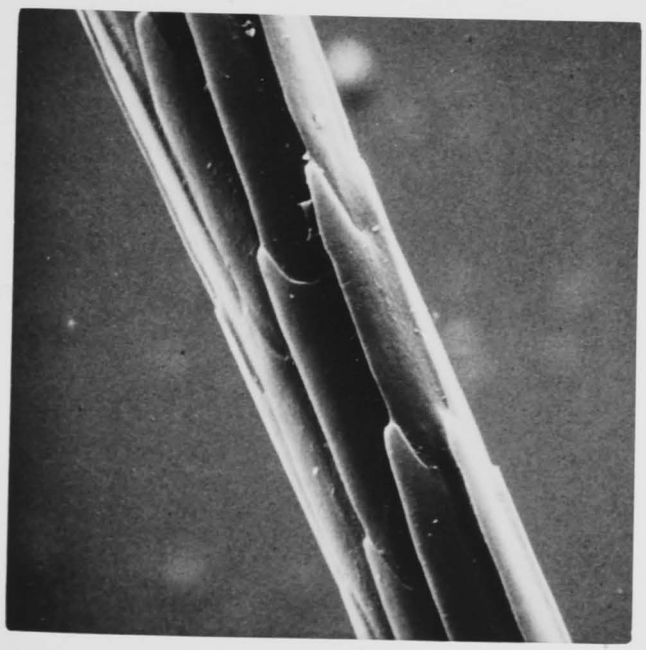
40μ

Figure 2.93. Effect of Chlorine Water on Platypus Belly Guard Hair - Mid-shaft.



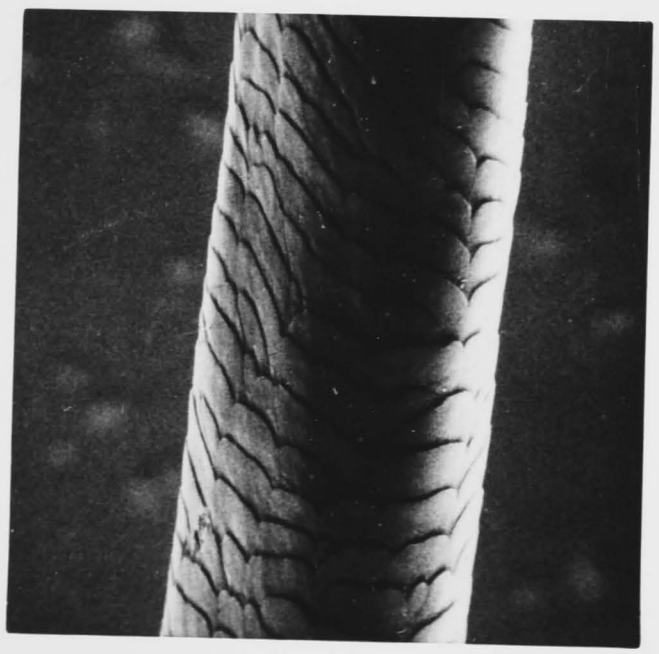
20μ

Figure 2.94. 'Stereoscan' of Platypus Belly Guard Hair - Basal.



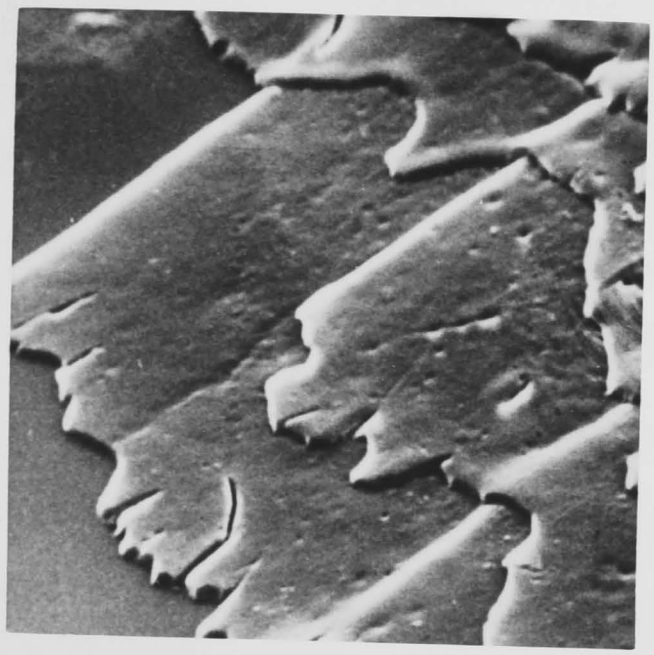
10μ

Figure 2.95. 'Stereoscan' of Platypus Belly Guard Hair - Mid-shaft.



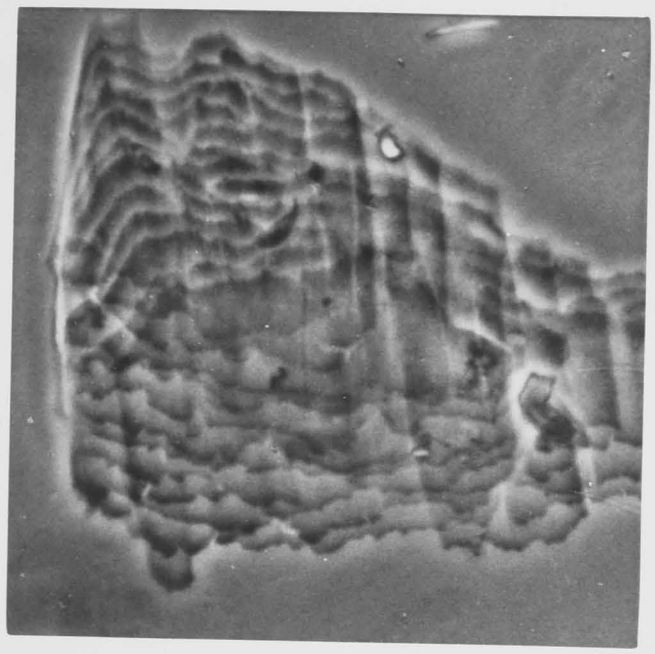
10μ

Figure 2.96. 'Stereoscan' of Platypus Belly Guard Hair - Distal.



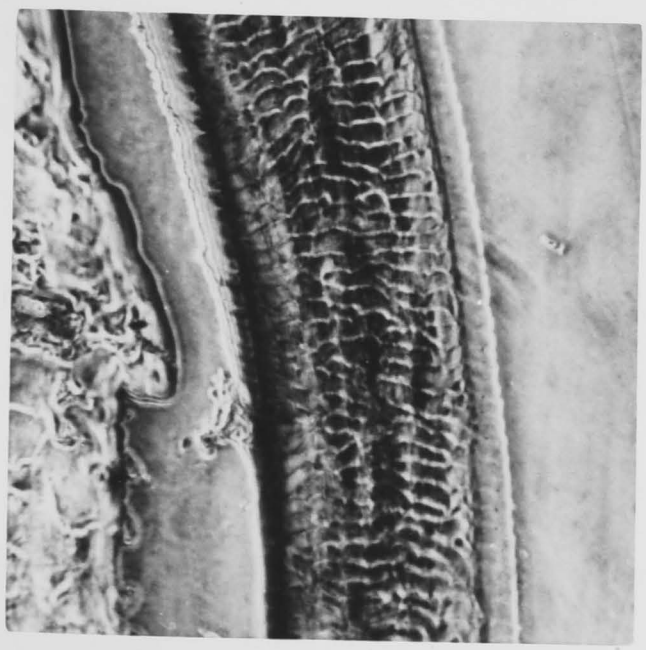
2μ

Figure 2.97. 'Stereoscan' of Cuticle Cells Isolated from Platypus Tail Guard Hair by HCOOH-treatment.



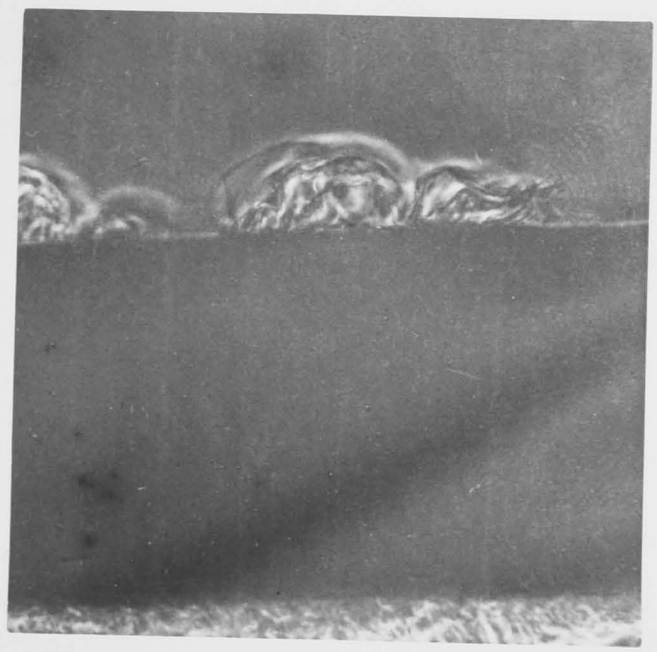
20μ

Figure 2.98. Cuticle Sheet Isolated from Platypus Tail Guard Hair by Two-stage HCl-HCOOH Treatment.



40μ

Figure 2.99. Platypus Tail Guard Hair -'Sellotape' Mount.



40μ

Figure 2.100. Effect of Chlorine Water on Platypus Tail Guard Hair, Partially Descaled by HCOOH-treatment.

The results described above are entirely consistent with those in section 2[E](i) concerning the association of epicuticle with the individual cuticle cells. In fact, several of the observations provide additional evidence in support of the 'discontinuous epicuticle' concept.

The detailed studies of size, shape and Allworden sac pattern, on isolated cuticle cells and whole fibres, also permits some deductions regarding the relation between the true cuticle cell boundaries and the 'scale edges' seen, e.g., on fibre profiles, surface replicas, or the intact cuticle sheets studied during the present work.

These visible scale ridges or transverse striations are formed in the follicle during fibre differentiation. Among the several histological layers of the follicle, only that nearest the formative fibre, the so-called 'inner root sheath', is pertinent to this discussion.* The inner cuticle layer of the inner root sheath is made up of scales, which point downward and interlock with similar but opposing scales on the surface of the fibre.

At approximately one-third of the way up the follicle, the fibre cuticle begins to harden during the keratinisation process, while the inner root sheath cuticle keratinises at a lower level in the follicle. Fibre and inner root sheath

* [For a fuller description of the histology and biochemistry of the hair follicle, see Auber (1952), Birbeck and Mercer (1957), Rogers (1964) and Straile (1965)]

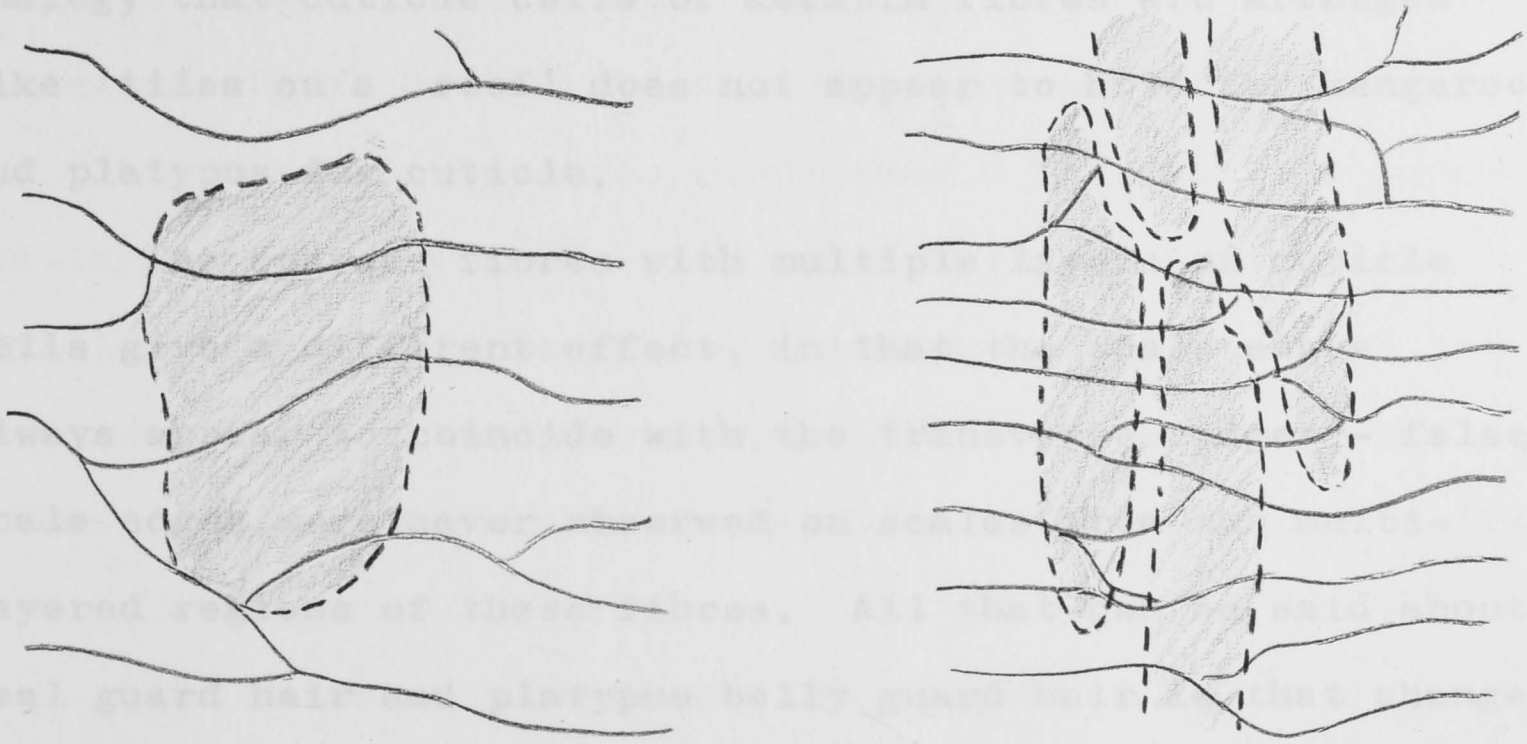
move up the follicle together, with their cuticles interlocked. Approximately half-way up the follicle the inner root sheath begins to disintegrate and/or dissolve, leaving the fibre to emerge unrestricted from the skin.

The cuticle of the inner root sheath hardens before that of the fibre, so must be responsible for moulding the surface structure of the wool or hair. Birbeck and Mercer (1957) interpret this process as a "shearing of the cuboidal cells into the imbricate condition seen in the final cuticle". Thus it would seem that transverse striations or ridges observed on keratin fibres are scale edges, but probably represent the scale edges of the inner root sheath rather than of the keratin fibre itself.

Up to this point, in accordance with common practice, these transverse ridges have been referred to as scale edges or false scale edges. This terminology appears to be more-or-less correct for Merino 64's fibres, where electron microscopy on longitudinal sections has shown that most ridges coincide with the distal or upper edge of a cuticle cell [see, e.g., Dobb et al. (1961); Bradbury et al. (1963)]. During studies of the growth rate of fine wool fibres, Rougeot (1965) concluded that the number of ridges was directly proportional to the number of transverse boundaries of the fibre cuticle cells. This also follows from the work of Kassenbeck (1958). The present results support this idea,

and indicate that only 50% of cuticle cells have transverse ridges which do not constitute cell boundaries.

On the other hand, two examples have been given above where there is no apparent relation between the transverse surface ridges and the boundaries of the cuticle cells. Cuticle cells from the distal end of platypus fur contain an average of 10 transverse ridges per cell, while some scales contain as many as 16 'false scale edges'. Kangaroo fur has 2-3 of these ridges per cuticle cell. Further, in both cases the ends of the cuticle cells do not necessarily coincide with a transverse ridge. These considerations suggest that the arrangements of cuticle cells in the fibre surfaces are similar to those shown in figure 2.101.



Kangaroo fur cuticle Platypus fur cuticle (distal)

Figure 2.101. Arrangement of Cuticle Cells in the Fibre Surface (Schematic).

Since the inner root sheath provides the template or mould which induces ridge formation on the fibre cuticle, the imprints of this sheath need not necessarily coincide with the boundaries of the flattened cuticle cells. The scanning electron micrograph in figure 2.86 (page 76a) suggests that platypus fur cuticle cells butt together longitudinally and transversely to form a coherent external layer.

As far as is known, the present work provides the first demonstration of the effect described above. More work needs to be done before this type of relation can be regarded as a general phenomenon. The effect will obviously vary for fibres of varying scale pattern, but the oft-quoted analogy that cuticle cells of keratin fibres are arranged like 'tiles on a roof' does not appear to hold for kangaroo and platypus fur cuticle.

The coarser fibres with multiple layers of cuticle cells give a different effect, in that the scale edges always appear to coincide with the transverse ridges - false scale edges were never observed on scales from the multi-layered regions of these fibres. All that can be said about seal guard hair and platypus belly guard hair is that changes in the cuticle pattern of the inner root sheath and in the cross-sectional shape of the follicle must have occurred in order to produce the gross variations in scale pattern and

diameter along the length of these fibres.

Width measurements on single scales also indicate that the number of cells around the circumference of a fibre cannot be expressed in terms of visible scale markings. Figure 2.39 (page 62a) is a sheet of human hair cuticle 200μ wide, so will accommodate approximately 7 cuticle cells of width 30μ, yet the transverse scale edges appear to be continuous (see also the 'Stereoscan' of an intact fibre - figure 2.38). This applies to all fibres studied, with the possible exception of those with scale structures resistant to disruption.

The shapes of the individual cuticle cells can be correlated with the ease of disruption of the cuticle layer. Many Merino 64's cuticle cells tend towards complete encirclement of the fibre, so resist disruption more than, e.g., the narrower kangaroo fur scales. The multiple layers and large degree of overlap of guard hair and human hair cuticles means that the scales are very firmly cemented in place on the untreated fibre, but after disruption of the intercuticular cement with formic acid, single cuticle cells readily separate.

The resistance to disruption exhibited by several of the fibres studied is probably a function of intercellular interactions of some form, but no detailed explanation can be advanced for this resistance.

With regard to the separation of intact cuticle sheets by the two-stage HCl-HCOOH treatment, the preferential separation of sheets rather than single cuticle cells suggests a difference in intercellular bonding. In this connection, Rogers (1959) showed by electron microscopy on cross-sections that the intercellular space between cuticle and cortex was slightly different from that between cortical or cuticle cells. This intercellular layer between cuticle and cortex is apparently preferentially attacked during the hydrochloric acid pretreatment.

2[E] (iii) Effect of Pretreatments on Sac Formation:

When wool fibres were immersed in freshly-prepared saturated chlorine water, time for appearance of Allworden sacs was found to be remarkably reproducible, usually varying not more than $\pm$ 1 second for unmodified Merino fibres. Full development of the sacs was essentially complete within 5 seconds of the start of sac formation. There was no detectable change in size or shape of the Allworden sacs once this initial rapid development was complete.

A typical equilibrium condition on untreated wool was shown in figure 2.2, page 14a.

The effect of saturated bromine water was not so

reproducible - uneven development of sacs occurred, both along individual fibres and between different fibres. This probably reflects the greater thickness of the Herbig membrane [King and Bradbury (1967)]. It may also be influenced by the possible difference in reaction mechanism suggested by the behaviour of human hair when treated with bromine water (see section 2[E](ii)).

On unmodified Merino fibres, chlorine-induced sacs appeared after 15±1 seconds, while bromine-induced sacs took between 30 and 50 seconds to appear. Thus the above factors, plus a probable difference in reactivity of the two halogens towards proteins, results in slower response of Merino fibres towards bromine, despite the much greater concentration of saturated bromine water - 1.3M or 10%w/w compared with 0.2M or 0.7%w/w for saturated chlorine water at 20°C.

The time taken for the first sacs to appear, and the manner in which these sacs approach their equilibrium condition, both depend on the type of pretreatment applied to the fibre. The effects of various chemical and physical modifications are listed in the following tables.

(a) Pretreatment with organic solvents:

Table 2.1 summarises the effects of treating Merino fibres with organic solvents prior to addition of chlorine or bromine.

TABLE 2.1. EFFECT OF SOLVENT-EXTRACTION TREATMENTS ON THE ALLWORDEN AND HERBIG REACTIONS

Treatment	Reaction time (sec)		Observations
	Chlorine	Bromine	
Untreated	15	30-50	Normal reaction (see figures 2.2 and 2.3, page 14a).
EtOH-extraction, 20°C, 5 hr.	15	30-50	Normal reaction.
EtOH-extraction, 70°C, 5 hr.	11	25-35	Increased rate of sac development.
EtOH-extraction, 70°C, 100 hr.	18	50-70	Normal reaction.
EtOH-extraction, 100°C, 24 hr.	22	70-100	Slower sac development.
HCOOH-extraction, 70°C, 5 hr.	17	15-25	Sac shape varied; sacs formed on partially-detached scales (see figure 2.5, page 37a).

The analyses reported by King and Bradbury (1967) have confirmed the protein nature of epicuticle; the small amount of lipid material found by these workers was probably a contaminant and would not be expected to contribute to the mechanism of sac formation. Thus, solvent treatments produced only small variations in the Allworden and Herbig reactions.

Ethanol extraction for a short time at 70°C may have enhanced sac formation by cleaning the fibre surface of wool-wax impurities, while the more severe ethanol extractions retarded development, probably because of degradation of the wool (the sample treated in ethanol at 100°C was badly discoloured).

The present results do not agree with those of Swift and Holmes (1965), who claimed that hot ethanol extraction removed epicuticle from human hair, "thereby demonstrating that epicuticle is made up of lipid-type material". Further, although formic acid extracts lipid from wool fibres [Bradbury et al. (1965a)] the characteristics of the Allworden and Herbig reactions were not greatly modified (table 2.1).

(b) Pretreatment with chemical shrinkproofing reagents:

Shrinkproofing treatments which involve chemical degradation are generally believed to depend, for their effectiveness, on removal or disruption of the epicuticle

[see, e.g., Gralen (1950); Lindberg (1953a,b); Stewart and Whewell (1960)]. To quote from a recent shrinkproofing review [Preston (1966)] - "All shrinkresist processes using any degradative attack on the scales disrupt epicuticle (and make wool easier to dye) ----".

The treatments listed in table 2.2, with the possible exception of the potassium hydroxide/salt sample, produce a commercially-acceptable degree of shrinkresistance, yet in every case the epicuticle was still present and still functioned as a semi-permeable membrane. In fact, sacs formed more readily on the oxidative-shrinkproofed samples (bromate/salt and permanganate/salt).

Stewart and Whewell (1960) regarded the extent of occurrence of Allworden sacs as a measure of the completeness of the epicuticular sheath. They reported that all shrinkproofing treatments severely reduced the extent of sac formation, so concluded that removal of epicuticle was a prerequisite for effective shrinkproofing.

The reason for the difference between the present results and those of Stewart and Whewell is not immediately obvious. Their alcoholic alkali treatment was with 2% potassium hydroxide for 30 minutes compared with the present treatment with 5.6% alkali for 5 minutes; perhaps Stewart and Whewell did not wait long enough for sac formation to occur. Thus Schuringa et al. (1952a) reported that the

TABLE 2.2. EFFECT OF SHRINKPROOFING TREATMENTS ON THE ALLWORDEN AND HERBIG REACTIONS.

Treatment	Reaction time (sec)		Observations
	Chlorine	Bromine	
Untreated	15	30-50	Normal reaction.
2% $KBrO_3$/satd.KCl	5	40-60	Sacs slightly smaller than normal.
5% $KMnO_4$/satd.KCl	9	12-18	Uneven sac development - up to 30 min. for full development.
1% thioglycollic acid/EtOH	15	40-80	Chlorine - normal reaction. Bromine - very uneven reaction.
10% SO_2Cl_2/CCl_4	14	30-50	Normal reaction.
5.6% KOH/EtOH	18	60-120	Chlorine - normal reaction. Bromine - uneven reaction.
5.6% KOH/satd.KCl	18	80-120	Chlorine - normal reaction. Bromine - slow development and very uneven coverage.

Allworden reaction was slow rather than negative for alkali-treated wools, indicating that the epicuticle was not disrupted by the treatment. Their results for rate of sac formation on fibres pretreated with 2% alcoholic alkali are reproduced in figure 2.102.

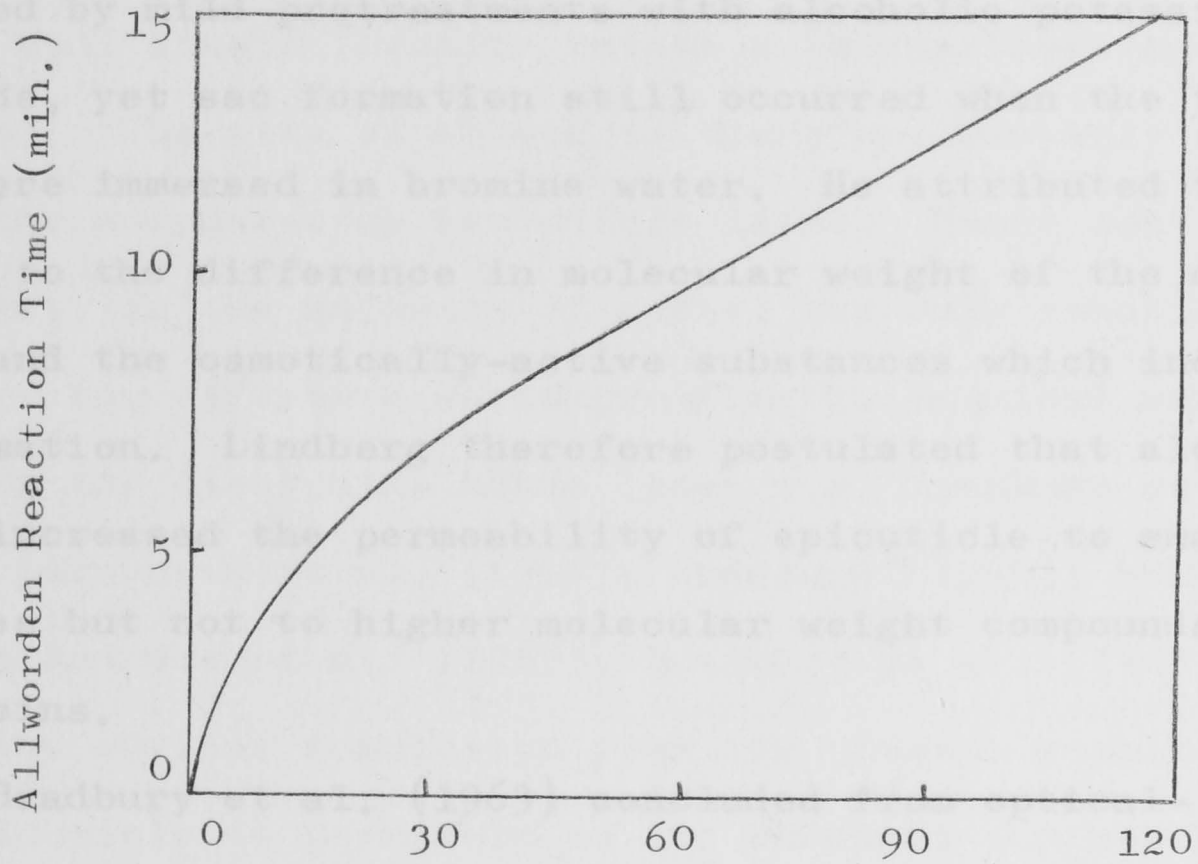


Figure 2.102. Effect of Pretreatment with Alcoholic Potassium Hydroxide on Allworden Sac Formation [from Schuringa et al. (1952a)].

Although the present results and those of Schuringa et al. (1952a) indicate a negligible degree of attack on the epicuticular membrane, changes in the wool obviously occur during surface shrinkproofing treatments. Andrews et al. (1963, 1966) report considerable changes in the amino acid

analyses of cuticle after oxidative shrinkproofing treatments, while the permanganate/salt and bromate/salt treatments used in the present work greatly increase the wettability and rate of dyeing of the wool [McPhee (1960b)]. Lindberg (1953a) found that the rate of uptake of acids and dyestuffs was increased by mild pretreatments with alcoholic potassium hydroxide, yet sac formation still occurred when the treated wools were immersed in bromine water. He attributed these effects to the difference in molecular weight of the dyestuffs and the osmotically-active substances which induce sac formation. Lindberg therefore postulated that alcoholic alkali increased the permeability of epicuticle to small molecules but not to higher molecular weight compounds such as proteins.

Bradbury et al. (1963) concluded from optical- and electron-microscopic studies that treatments similar to those listed in table 2.2 produced severe surface modifications on wool fibres. Thus the efficacy of chemical shrinkproofing treatments correlates well with the severity of surface modification observed under the microscope [Bradbury (1960, 1961)]. Ramanathan et al. (1955) studied replicas of fibre surfaces under the electron microscope, and demonstrated that severe pitting and erosion of the exterior of the fibres resulted from surface-limited treatments (notably a sulphuryl chloride treatment of

unspecified extent). This is not consistent with the present observations of intact epicuticle or Lindberg's (1953a) postulate of membrane permeability.

The greater variations in rate and extent of sac formation for bromine water suggests that attack by the shrinkproofing reagent has occurred somewhere within the cuticle cell itself (bromine raises a thicker membrane than chlorine). The site of attack is therefore probably on or within the sulphur-rich exocuticle layer. Hence most workers agree that, in the majority of cases, the only reaction necessary for effective shrinkproofing by chemical means is attack on the disulphide bonds [see, e.g. Speakman et al. (1938); Farnworth et al. (1949); Bradbury (1960); McPhee (1960a); Andrews et al. (1963); Bradbury et al. (1963)].

The obvious conclusion from the present results is that epicuticle is permeable to the chemical shrinkproofing reagents and reaction by-products yet remains impermeable to the chlorine and bromine reaction by-products (or at least to a sufficient proportion of the halogen reaction by-products for sac formation to occur).

Support for this conclusion is given by the report of Makinson (1968) that permanganate/salt treatment degrades the protein <u>inside</u> the scales, leaving epicuticle intact.

A comparative study of the size and amino acid composition of the degradation products which pass through the epicuticular membrane and those which are held back,

should prove to be of interest. Some preliminary experiments along these lines are reported in chapter 4 of this thesis.

(c) Pretreatments under non-swelling conditions

On unmodified wool, the rate of penetration of non-polar solvents or polar solvents of molecular size greater than n-propanol, is very slow if wool and solvent are carefully and thoroughly dried; and the rate decreases further with increasing chain-length and/or degree of branching [Bradbury and Leeder (1963)]. This knowledge has been utilised to develop methods for selectively removing impurities and contaminants from the fibre surface, using such solvents as dry tert-butanol and dry 2,2,4-trimethyl pentane [Anderson and Leeder (1965a,b)]. Surface area studies have also shown that penetration of dry carbon tetrachloride is extremely slow [Brooks and Leeder (1967)].

The absorption results summarised above suggest possibilities for confining chemical reactions to the fibre surface. The treatments listed in table 2.3 are representative of a great many attempts to selectively remove epicuticle by surface-limited chemical action.

The fact that epicuticle was still intact after refluxing wool with 1M potassium tert-butoxide for 3 hours, or treatment with 100% sulphuryl chloride at 40°C for 70 hours (table 2.3), again demonstrates the extreme chemical inertness of the outer membrane. The Allworden reaction

TABLE 2.3. EFFECT OF ANHYDROUS SURFACE TREATMENTS ON THE ALLWORDEN AND HERBIG REACTIONS

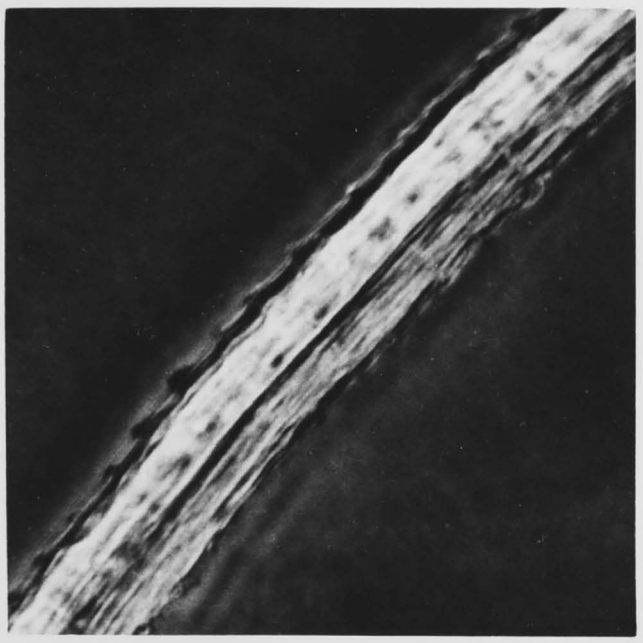
Treatment	Reaction time (sec)		Observations
	Chlorine	Bromine	
Untreated	15	30-50	Normal reaction.
tert-butanol extraction	13	30-50	Normal reaction.
1M pot. tert. butoxide	16	25-35	Normal reaction.
50% SO_2Cl_2/CCl_4	30	30-60	Very small sacs formed.
100% SO_2Cl_2	1-2 hr.	No reaction	Chlorine - very slow sac development (see figure 2.103); nearly-complete reaction after 16 hr. Bromine - no reaction detected after 16 hr.

occurred to only a limited extent following the sulphuryl chloride treatment, but figure 2.103 shows incipient sac formation at most scale tips. This demonstrates that epicuticle is still present and is 'trying' to form sacs. Full development is probably hindered because the exocuticle has been modified in such a way that it is not attacked by chlorine in the manner of untreated wool.

Although no weight change was observed after the prolonged anhydrous sulphuryl chloride treatments, examination of the treated fibres under the scanning electron microscope (figure 2.104) reveals considerable surface modification. In addition to puckering of the fibre surface, note that the scales are beginning to separate from the rest of the fibre. The fact that sac formation occurs on such a fibre provides further evidence that epicuticle cannot be continuous over the fibre surface.

Treatment with potassium tert-butoxide produced no detectable change in weight of the wool, or in Allworden sac formation (table 2.3), or in the surface of the fibres as seen under the scanning electron microscope (figures 2.105 and 2.106). However, the handle or feel of the treated wool was considerably harsher than that of untreated, indicating that some type of chemical (or physical) attack did result from the treatment.

When loose bundles of fibres treated with potassium



20μ

Figure 2.103. Effect of Chlorine Water on Merino Fibre Treated with 100% Sulphuryl Chloride,40°C-70 hr.

4μ

Figure 2.104. 'Stereoscan' of Merino Fibre Treated with Sulphuryl Chloride,40°C-70 hr.

Figure 2.105. 10μ

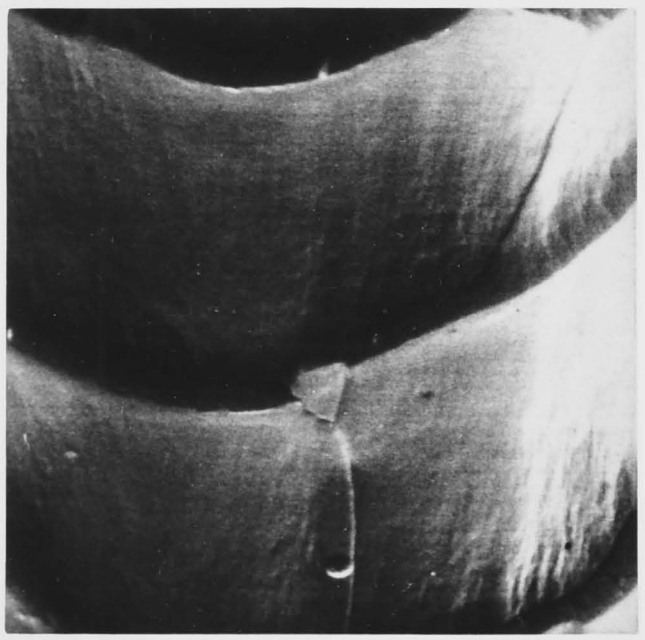


Figure 2.106. 2μ

'Stereoscans' of Merino Fibres Treated with 1M Potassium tert-Butoxide under Reflux for 3 hr.

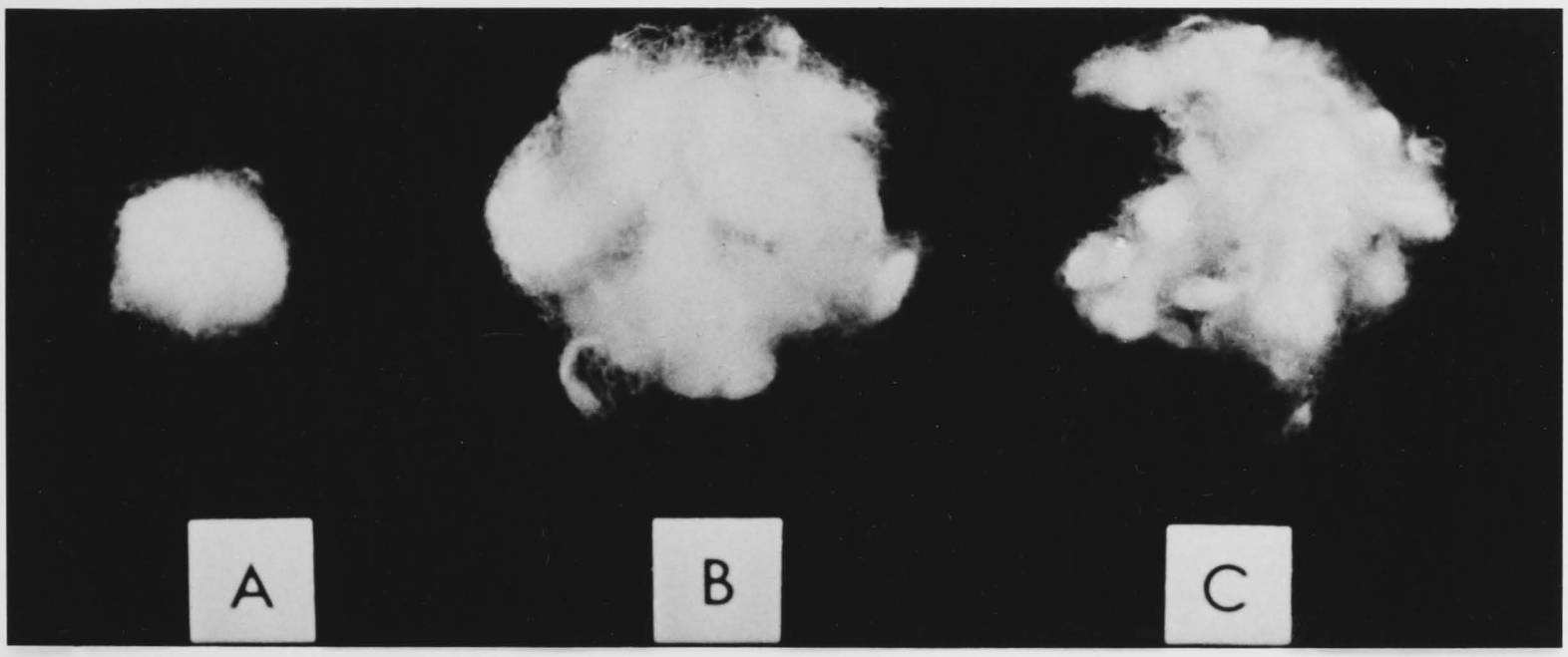
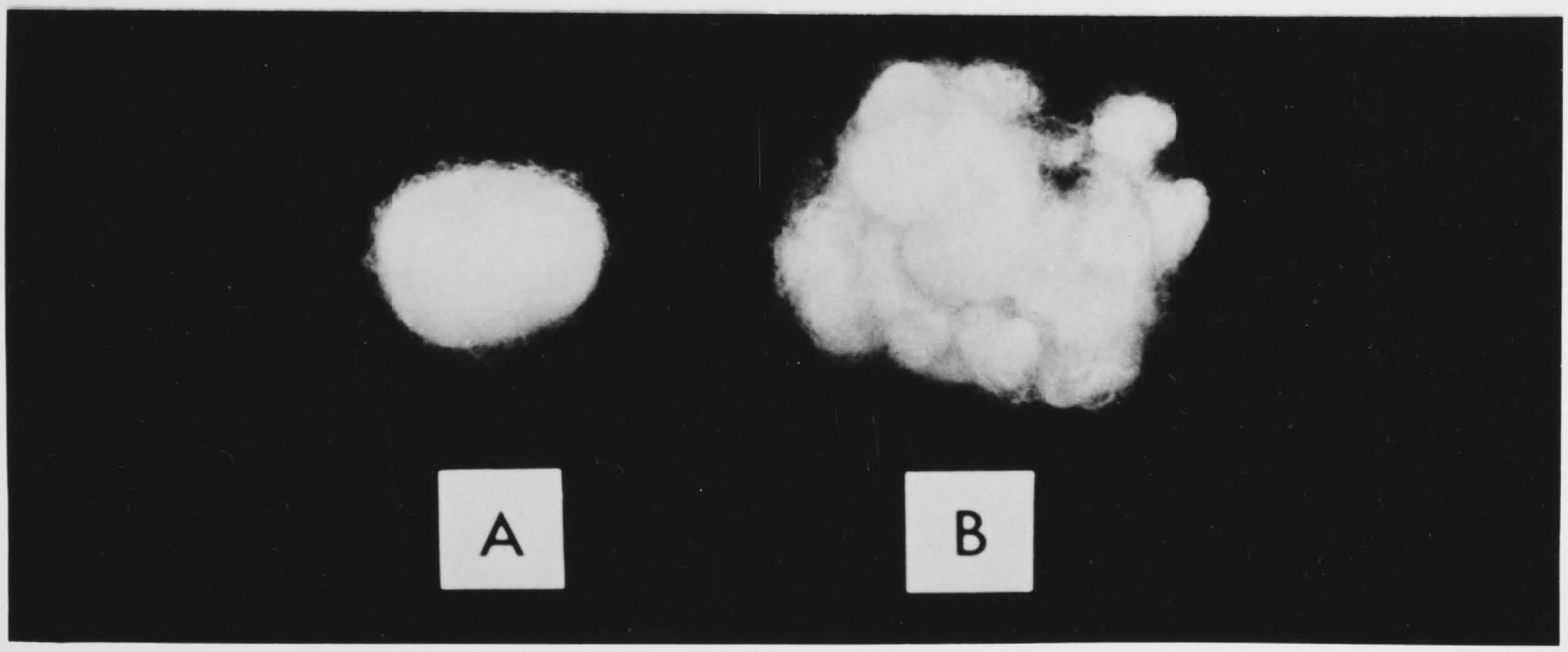


Figure 2.107. Wool Samples after Shaking in Soap Solution.

Figure 2.108. Wool Samples after Shaking in Soap-Soda Solution.



(A) - Untreated,

(B) - Treated with Potassium tert-Butoxide,

(C) - Treated with Permanganate/Salt.

tert-butoxide were subjected to a qualitative shrinkproofing test (i.e. agitation for several hours in dilute aqueous soap or soap-soda solutions) a degree of shrinkresistance similar to that of permanganate/salt treatment was found! (see figures 2.107 and 2.108). Treatment of wool with potassium tert-butoxide under anhydrous conditions is, of course, the ultimate extension of the alcoholic alkali shrinkproofing treatment developed by Freney and Lipson in 1940. The use of an organic solvent restricted the action of the alkali to the fibre surface, producing good shrink-proofing with minimal side-effects.

The shrinkresistant effect obtained with potassium tert-butoxide may shed some light on the incompletely-understood mechanism of shrinkproofing by degradative chemical means. Unlike the dry sulphuryl chloride treatments, there was no evidence of attack within or between the scales. This treatment therefore seems to be an exception to the 'rule' that degree of shrinkresistance depends on degree of visual surface modification [Bradbury (1960,1961); Bradbury et al. (1963)].

The only other possible exception found by Bradbury was treatment with ethanolic alkali, which was assumed to function by removing a wool-wax constituent from the cell membrane complex beneath the cuticle. The zero weight loss and undisturbed scale structure (figures 2.105 and 2.106)

indicate that this mechanism is not operative for the potassium tert-butoxide treatment.

Changes in frictional properties are usually found after chemical shrinkproofing treatments [see, e.g., Alexander (1950); Gralen (1950); McPhee (1960a); Bradbury (1961)]. Untreated wool is considered to felt or shrink during agitation in aqueous media because of a difference in the 'with-scale' and 'against-scale' frictional coefficients. This difference is called the Directional Frictional Effect, usually abbreviated to D.F.E.

Both the with-scale and against-scale frictional coefficients of wool are increased by alcoholic alkali treatments, but the D.F.E. remains almost unchanged [Lipson (1947); Gralen (1950); Bradbury (1960)]. The altered handle of wool treated with potassium tert-butoxide suggests that the frictional coefficients are modified, but this change (and/or whatever other changes produce the shrink-proofing effect) apparently must occur exclusively in the 30Å-thick epicuticle!

Lindberg and Gralen (1950) and Lindberg (1953b) suggest that increases in the coefficients of friction for wools treated with alkali in organic solvents may result from formation of polar groups in the epicuticle or by the uncovering of such groups in the structure underneath. From data on changes in wettability of wool fibres after

treatment with alcoholic alkali, Lindberg (1953d) concluded that this treatment modified the molecular structure of epicuticle.

Lindberg and Gralen (1950) further consider that the with-scale frictional coefficient of wool fibres is much lower than the coefficients of most other textile fibres because of the chemical inertness of epicuticle. They report that this with-scale coefficient is close to that of polyethylene, indicating the presence of an inert (paraffinic?) surface layer.

The above considerations therefore suggest another possible mechanism for the shrinkproofing effect obtained with potassium tert-butoxide - this reagent, being an excellent degreasing reagent, may cause the desorption and/or dissolution of strongly-bound lipid material, resulting in a clean but rough surface. A very thin layer of lipid could, in theory, originate from the protein-lipid interface of the unit cell membrane (see chapter 3).

Closer studies of possible mechanisms for the 'chemical shrinkproofing without chemical damage' observed with potassium tert-butoxide treatment under anhydrous conditions, may lead to a better understanding of shrinkproofing reactions in general, and of the role of epicuticle in the felting of wool fibre assemblies.

(d) Whole-fibre chemical pretreatments:

The changes in Allworden and Herbig sac formation following several whole-fibre modifications are summarised in table 2.4.

When wool is heated for extended times in dilute hydrochloric acid, dissolution of the orthocortex and the endocuticle occurs [Leach et al. (1964)]. Whether this has any connection with the greatly increased rate of reaction with chlorine or bromine, or whether partial hydrolysis of exocuticle proteins is responsible for this effect, is not apparent at this stage. The changes were more pronounced with the bromine reaction, suggesting that attack has occurred deeper within the cuticle cells than the outermost layers of the exocuticle.

Conversely, treatments with alkalies (potassium hydroxide and ammonia) decrease the rate of sac formation.

These observations are in agreement with those of Meeuse et al. (1950) and Lindberg (1949, 1950, 1953a) using bromine water, and of Leveau and Cebe (1953) using chlorine and bromine. Lindberg (1953a) suggested that the reduced reaction rate found with alkali-treated wools was due to stabilisation of the protein underlying the epicuticle by formation of lanthionine cross-links.

In the present work, it was found that wool pretreated with potassium cyanide gave no indication of sac formation

TABLE 2.4. EFFECT OF WHOLE-FIBRE CHEMICAL TREATMENTS ON THE ALLWORDEN AND HERBIG REACTIONS.

Treatment	Reaction time (sec)		Observations
	Chlorine	Bromine	
Untreated	15	30-50	Normal reaction.
Peptide hydrolysis	10	7-10	Sacs slightly 'fuller' than normal.
5.6% aqueous KOH	21	40-60	Normal reaction.
conc. ammonia	120	40 minutes	Few small sacs slowly develop.
potassium cyanide	no reaction	no reaction	No reaction after 6 weeks contact with halogen.
hyaluronidase	30 minutes	no reaction	Chlorine - sacs slowly reached normal development. Bromine - no reaction after 18 hrs.
peracetic acid	20	100-150	Sacs smaller than normal and slower to develop.
mercuric acetate	8	18-20	Normal reaction.
phosphotungstic acid	18	100-150	Slower sac development.
pronase	15	16-20	Slow development - scales lifted from fibre surface with bromine, and fibres began to disintegrate.

even after immersion in chlorine water or bromine water for as long as 6 weeks. This reagent converts cystine to lanthionine [Crewther et al. (1967)]. There was no visible evidence of attack by the halogen solutions after these long-term immersions, supporting the 'lanthionine-stabilisation' theory. This is in contrast to the change in scale structure observed when unmodified wool is treated with chlorine water (see figure 2.24, page 51a).

Treatment with hyaluronidase, a mucolytic enzyme, was included in the present study because of a report that this enzyme produced "some evidence of attack" on epicuticle [Lagermalm et al. (1951)]. Rate of acid sorption, however, was not altered by the treatment of Lagermalm et al. The very slow Allworden reaction rate shown in table 2.4 indicates that some type of modification occurred, but the eventual formation of sacs suggests that this was another example of conversion of cystine to lanthionine by alkali - the treatment was done at pH 8.4 and 40°C for 9 weeks.

Peracetic acid destabilises the molecular structure of proteins by oxidising cystine cross-links to cysteic acid, yet the Allworden and Herbig sacs were found to have a slower, rather than an anticipated faster, rate of formation.

Mercuric acetate is considered to form -S-Hg-S- cross-links in wool [Barr and Speakman (1944; Anglis (1965);

Leeder (1965)] but sac formation occurred at a faster rate with both chlorine and bromine. This suggests that the -S-Hg-S- linkages are more susceptible to halogen attack than the original disulphide cross-links.

The results obtained after whole-fibre treatments have a bearing on the mechanism of Allworden and Herbig sac formation - discussion of this aspect will be deferred until section 2[E](iv).

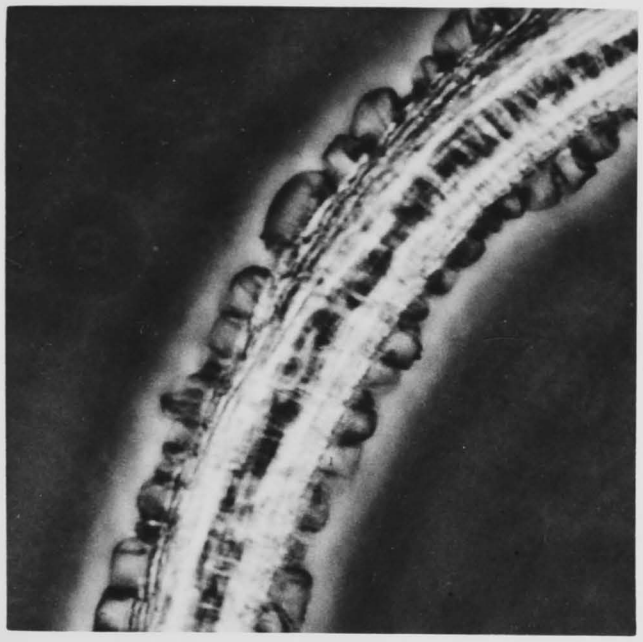
Treatments with phosphotungstic acid and with the proteolytic enzyme, pronase, are of interest in that the rate of reaction with bromine water is changed to a much greater extent than that with chlorine water. This effect will also be discussed in section 2[E](iv).

(e) Effect of some physical factors on sac formation:

Observations of the action of chlorine and bromine on various physically-modified Merino fibres are listed in table 2.5, illustrated by figures 2.109 - 2.112.

Association of epicuticle with individual cuticle cells is suggested by photographs 2.109 and 2.110, which depict crowding and elongation of sacs on supercontracted and stretched fibres, respectively. No other changes were observed.

Sac formation was not affected by the presence of the large amounts of grease and dirt (greasy wool-figure 2.111), giving another indication that the chemical nature of



20μ

Figure 2.109. Effect of Chlorine Water on Supercontracted Merino Fibre.

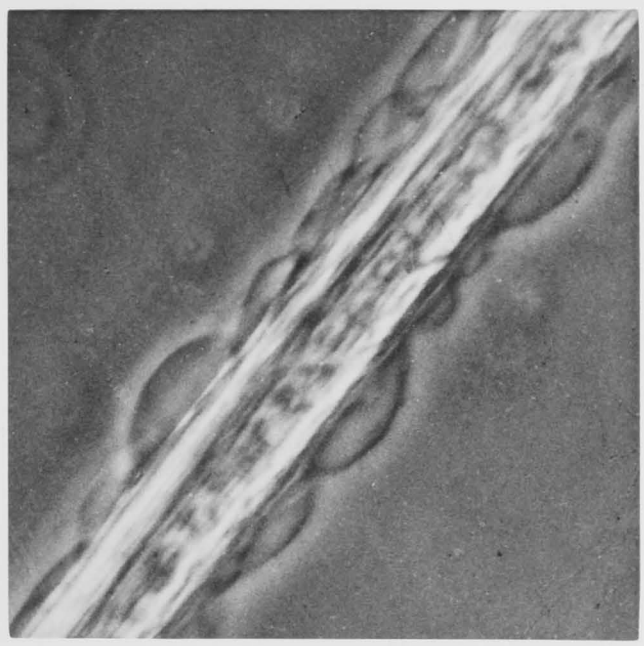


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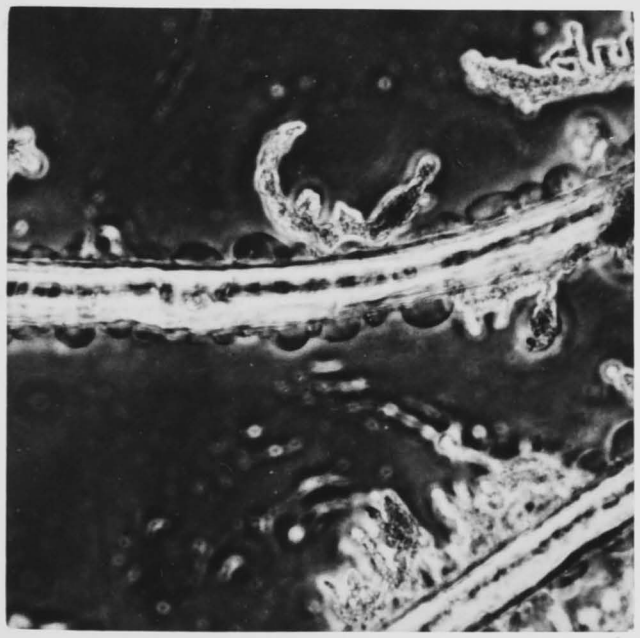
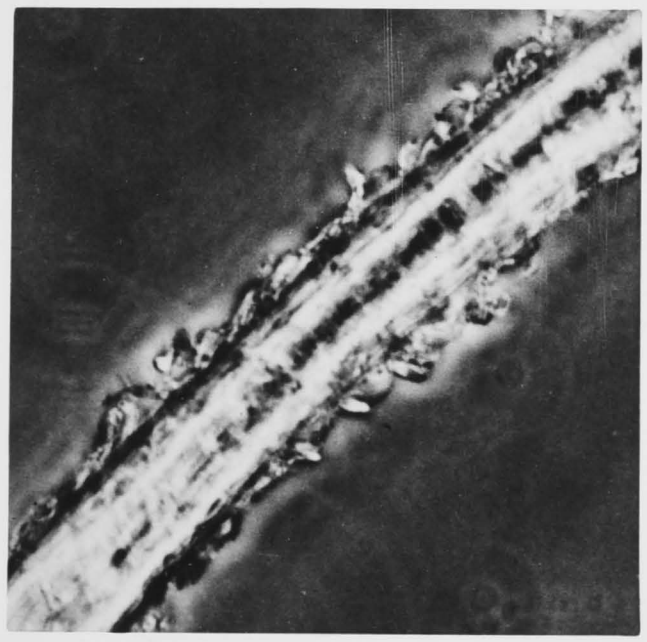
Figure 2.110. Effect of Chlorine Water on Stretched Merino Fibre.



40μ

Figure 2.111. Effect of Chlorine Water on Greasy Merino Wool.



20μ

Figure 2.112. Effect of Chlorine Water on Abraded Merino Wool.

TABLE 2.5. EFFECT OF SOME PHYSICAL FACTORS ON THE ALLWORDEN AND HERBIG REACTIONS.

Sample	Reaction time (sec)		Observations
	Chlorine	Bromine	
Untreated	15	30-50	Normal reaction.
Supercontracted fibres	15	30-50	Sacs 'crowded' (see figure 2.109)
Stretched fibres	14	30-50	Sacs 'elongated' (see figure 2.110)
Greasy wool	18	35-55	Normal reaction - sacs formed under layers of dirt and grease (see figure 2.111)
Tip wool, EtOH-extracted	15+	30+	Sacs decreased in size and increased in time to appear and time to develop as the tip end was approached.
Fibres from worsted fabric	16	40-90	Very uneven reaction - some fibres gave normal reaction, some fibres no reaction.
Fibres rubbed between fingers Fibres shaken with abrasive powder Powdered wool	15	30-50	Cuticle showed evidence of considerable disruption, but many sacs still formed. (see figure 2.112).

epicuticle is dissimilar to that of wool wax.

Variable results were obtained when the tip regions of Merino fibres were immersed in chlorine water or bromine water - reaction became progressively less pronounced as the tip end was approached. This was probably a result of weathering and sunlight irradiation. Thus Haly (1958) found that Herbig sacs formed much more slowly on wool after irradiation with ultra-violet light.

The extent of sac formation was greatly decreased by the mechanical processing necessary to produce a worsted fabric. This is in agreement with the views of Speakman (1950), but it is notable that about 50% of the epicuticle is still intact - Figure 2.112 shows limited sac formation on a fibre which had been rubbed between finger and thumb [after Whewell and Woods (1944)]. Again considerable amounts of epicuticle had been disrupted, but there is also evidence of loosening and separation of the cuticle cells, showing that the whole cuticle can be damaged <u>before</u> all the epicuticle has been disrupted. Similar effects were obtained for the other mechanically-damaged samples listed in table 2.5.

When it is considered that epicuticle is only about 30$\mathring{A}$ thick and forms the outer surface of keratin fibres, the above results suggest that it is perhaps a little unfair to accuse this membrane of being 'mechanically weak and

easily damaged by physical means', as many workers have done in the past.

2[E](iv) Mechanisms of the Allworden and Herbig Reactions:

Schuringa et al. (1953) consider that the conditions necessary for formation of Allworden sacs are, firstly, that the epicuticle should be intact to be able to function as a semi-permeable membrane, and, secondly, that the osmotic pressure of the substances active beneath the membrane should be strong enough to overcome the forces which cause the surfaces to adhere to each other.

The foregoing results and discussions in sections 2[E](i), (ii) and (iii), coupled with considerations of data from the literature, have established the following facts, most of which bear on the mechanism of sac formation -

(1) Epicuticle covers single cuticle cells, but -

(2) Sacs occur on only the top surface of each cuticle cell, and not at all on cortical cells.

(3) Epicuticle consists mainly of protein material.

(4) The osmotically-active substances which form under the membrane are protein in nature.

(5) The rate and extent of sac formation can be altered by chemical pretreatments, but -

(6) The epicuticle itself is extremely resistant to a wide range of chemical reagents.

(7) Cystine is probably involved in the chemistry of the reaction.

The following relevant observations were also made during the present work -

(8) Long-term experiments were carried out by sealing wool + chlorine water between a microscope slide and a cover slip with paraffin wax. The Allworden membranes slowly became thinner with increasing time of treatment, but most sacs retained their original dimensions for at least one week, after which they became too thin to be clearly seen. Similarly, the thicker membrane raised by bromine was still intact after 3 weeks, although in both cases the original scale structure appeared to be considerably modified. These results suggest that epicuticle is neither perforated nor easily dissolved by aqueous solutions of chlorine or bromine, and indicate that the osmotically-active substances originate in the exo- and/or endo-cuticle rather than the epicuticle.

(9) Bromine failed to induce sac formation on wool from which epicuticle had been removed by agitation in chlorine water. This demonstrates that intact epicuticle is essential for formation of Herbig sacs, and that the additional protein layer raised by bromine ('a' layer of the exocuticle?) is permeable to the material dissolved by bromine.

Two of the above points need to be considered in

greater detail before the mechanism of Allworden and Herbig sac formation can be fully described - viz. the extent of involvement of cystine, and the formation of sacs on only one surface of cuticle cells.

(a) Extent of involvement of cystine:

Examination of the data given in section 2[E](iii) shows that most of the chemical treatments which markedly affected time for appearance of Allworden sacs were those which modified cystine. The only possible exceptions were partial hydrolysis with dilute hydrochloric acid, severe ethanol treatments, and staining with phosphotungstic acid.

Treatments with potassium cyanide or alkalies decreased the reaction rate. These reagents convert disulphide cross-links (cystine) to monosulphide cross-links (lanthionine) and also induce formation of small amounts of lysinoalanine cross-links between the free amino group of lysine and a dehydro-alanyl residue originating from cystine [Ziegler (1965); Crewther et al. (1967)]. Thus, Lindberg (1953a) suggested that sac formation was retarded because of stabilisation of the underlying protein material by the formation of lanthionine. Hock et al. (1941a) found that reaction time increased with time of alkali treatment, while Mark (1925) observed the same effect with increasing concentration and/or temperature of alkali treatment. Similar observations were made by Meeuse et al. (1950) and

Leveau and Cebe (1953).

Sulphuryl chloride treatments, which also inhibit sac formation, result in conversion of $-CH_2.S.S.CH_2-$ cross-links to $-CH_2.Cl$ residues (in the absence of water), and to $-CH_2.SO_2.Cl + -CH_2.Cl$ in the presence of water [Hall (1939); Farnworth and Speakman (1949)].

Mercuric acetate treatment apparently makes the wool more reactive towards chlorine and bromine by converting -S-S- to -S-Hg-S-, resulting in a faster reaction rate. Normal behaviour towards halogens was restored by subsequent exposure of the mercury-containing wool to direct sunlight for several days. This was probably a result of breakage of the -S-Hg-S- cross-links by ultra-violet irradiation; the fibres turn black on irradiation due to mercuric sulphide formation [Leeder (1965)].

Oxidation of -S-S- to $-SO_3H$ by shrinkproofing treatments such as permanganate/salt and bromate/salt, increased the reaction rate, probably because of the greater polarity or charge of the oxidised protein. Surprisingly, the more-specific oxidation with peracetic acid resulted in a slower response of the treated wool to halogens. This suggests that conversion of cystine to cysteic acid by chlorine or bromine is not the only requirement for sac formation.

Osmotic effects cause formation of the Allworden and

Herbig sacs. Osmosis is the flow of solvent from a dilute solution to a more-concentrated solution through a semi-permeable membrane, and is therefore a 'solution' property.

These considerations lead to the following proposed general mechanism for the Allworden (or Herbig) reaction -

The chlorine (or bromine) diffuses through the epicuticle and reacts with the exocuticle component of the individual cuticle cells, causing hydrolysis of peptide bonds [Schuringa et al. (1953); Valk (1965)] and oxidation of cystine to cysteic acid [Valk (1965); King and Bradbury (1967)]. The resulting water-soluble peptides then induce sufficient osmotic forces for sac formation to occur.

When cystine is converted to a form which is not oxidised by halogen, the difference in concentration of solution on each side of the epicuticular membrane is reduced; this retards sac development. Conversely, prior degradation of the fibre by oxidation and peptide bond fission (or peptide hydrolysis alone) increases the reaction rate. Thus partial hydrolysis with hydrochloric acid prior to addition of halogen had this effect (table 2.4). The failure of peracetic-oxidation to increase the reaction rate highlights the importance of peptide hydrolysis in the proposed mechanism.

The role of 'oxidisable' cystine in sac formation was investigated further; the Allworden reaction was applied

to samples of wool in which known amounts of cystine had been reduced with tributyl phosphine and blocked with iodoacetic acid or ethyleneimine [Sweetman and Maclaren (1966); Maclaren and Sweetman (1966)] - these samples were kindly supplied by Dr. J. A. Maclaren and Mr. A. Kirkpatrick. Time for appearance of sacs is plotted against residual cystine content in figure 2.113.

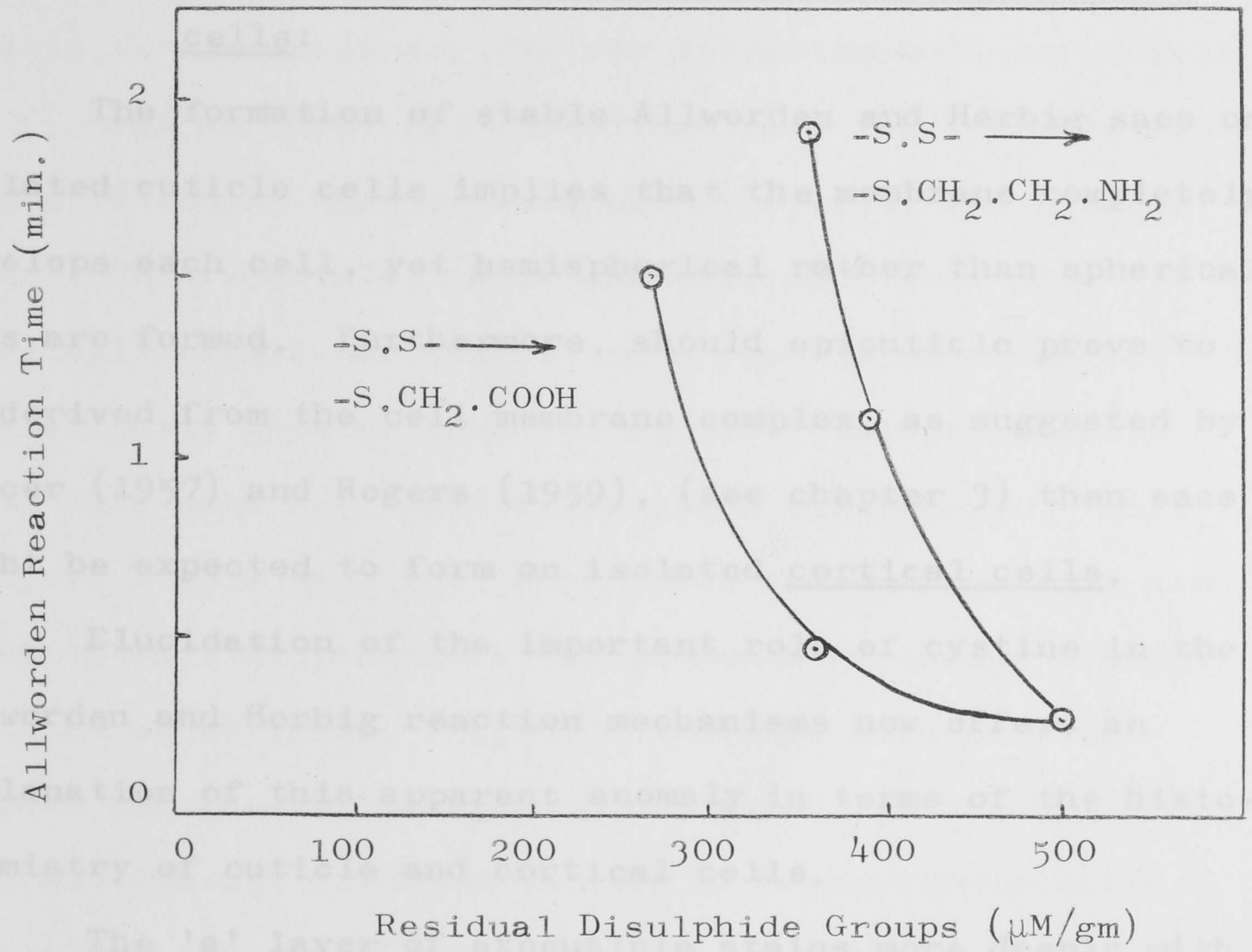


Figure 2.113. Relation between Residual Cystine Content and Time for Allworden Reaction on Reduced and Blocked Fibres.

These results show that conversion of only a small proportion of cystine, even to polar groups such as -S.CH_2.COOH and -S.CH_2.CH_2.NH_2, severely retards the rate of sac formation. If Nature had endowed keratin fibres with slightly less cystine, perhaps Allworden and Herbig would never have observed the phenomena which bear their names!

(b) Formation of sacs on only one side of cuticle cells:

The formation of stable Allworden and Herbig sacs on isolated cuticle cells implies that the membrane completely envelops each cell, yet hemispherical rather than spherical sacs are formed. Furthermore, should epicuticle prove to be derived from the cell membrane complex, as suggested by Mercer (1957) and Rogers (1959), (see chapter 3) then sacs might be expected to form on isolated cortical cells.

Elucidation of the important role of cystine in the Allworden and Herbig reaction mechanisms now offers an explanation of this apparent anomaly in terms of the histochemistry of cuticle and cortical cells.

The 'a' layer of exocuticle stains more deeply with metals than the other cuticle cell layers, so is considered to possess a higher proportion of sulphur [Dobb et al. (1961)] The greater concentration of (disulphide) sulphur in this region therefore explains why sacs form on the outside of scales. Electron microscope studies of metal-stained

cross-sections suggests that the underside of the cuticle cells (i.e. the endocuticle) has a very low cystine content (see also Appendix) so osmotically-active protein degradation products will not be produced in this layer, hence sac formation does not occur on the underside of cuticle cells. There is no evidence for the existence of coherent layers of sulphur-rich protein material adjacent to the membranes which surround the individual cortical cells, so again the osmotic forces necessary for sac formation will not develop.

King and Bradbury (1967) analysed the contents of sacs produced by contact with chlorine water and bromine water. Unfortunately, their analyses show that this osmotically-active substance has a cysteic acid content only slightly greater than the cystine content of whole wool; this does not support the above hypothesis. Their analyses also show a very high total sulphur content, some of which they attribute to the presence of sodium bisulphite (used as an antichlor reagent). Perhaps some of this sulphur was present as cystine oxides other than cysteic acid and was not detected because of concurrent elution with other amino acids from the chromatography column.

Andrews et al. (1966) reported that the cystine + cysteic acid content of Merino cuticle from chlorine-treated wools was less than that of unmodified cuticle material. Significant amounts of cystine were apparently converted to

forms other than cysteic acid and/or were lost from the fibre during treatment. These workers also found that the sulphur imbalance became greater when an antichlor aftertreatment was applied.

No further reasons can be advanced for the lack of correlation between the analytical figure for cysteic acid content of Allworden and Herbig sacs and the present demonstration that the presence of large quantities of oxidisable sulphur is essential for sac formation. Support for the view that oxidation of cystine to cysteic acid is important in the Allworden reaction, is provided by the work of Valk (1965), who found the cysteic acid content of protein material dissolved from wool by chlorine, to be almost double that of whole wool.

(c) Cystine content of cuticle versus Allworden reaction time for various keratin fibres:

Separation and analysis of cuticle material from a wide range of keratin fibres have been carried out by other workers in this department. In view of the relation between cystine content of Merino 64's cuticle and Allworden reaction time, these properties have been compared for the fibres listed in table 2.6.

The complete lack of correlation between cystine in the cuticle and Allworden reaction time for these fibres demonstrates that the physical structure of the cuticle cells

TABLE 2.6. TIME FOR ALLWORDEN REACTION VERSUS CYSTINE CONTENT (MOLE %) OF CUTICLE MATERIAL FROM VARIOUS KERATIN FIBRES

Fibre	Reaction time (sec)	Cystine Content	Reference for Cystine Analytical Data.
Merino 64's	15	14.4	King (1967)
Lincoln 36's	60	16.5	King (1967)
Kangaroo fur	15	18.2	O'Shea (1968)
Human hair	30-60	19.0	Chapman (1967)
Platypus fur	300-600	15.4	O'Shea (1968)
Possum fur	10-30	15.1	Chapman (1967)
Rabbit fur	45	14.1	King (1967)
White alpaca	5-15	17.9	Chapman (1967)
Brown alpaca	20	12.9	Chapman (1967)
Mohair	25	15.2	King (1967)
Bibrik wool	60	15.1	King (1967)

and/or of the whole fibres can greatly affect the response of each type of fibre to chlorine water. This does not suggest, of course, that susceptibility of the material underlying epicuticle to oxidation and peptide-bond hydrolysis are not the factors which control the mechanism of Allworden reaction for that particular fibre.

(d) Differences in mechanism of Allworden and Herbig Reactions:

Saturated bromine water is 1.3M and saturated chlorine

water 0,2M, yet chlorine-induced sacs form in 15 seconds and bromine-induced sacs in 30-45 seconds, on Merino wool [section 2[E](iii)]. This may result from the different sites of reaction within the exocuticle, since Allworden membranes are 30Å thick and Herbig membranes approximately 140Å thick. Diffusional effects due to the size of chlorine and bromine molecules and the degree of reactivity between the keratin and the halogen will also be contributing factors.

When chlorine was added to bromine-treated human hair, before and after extraction with hot formic acid (section 2[E](ii)) the effects obtained indicated that, as well as differences in the reaction of chlorine and bromine within individual cuticle cells, the presence of intercellular cement influenced the Herbig reaction more than the Allworden reaction.

A close study of the results tabulated in section 2[E](iii) show that 4 pretreatments alter the rate of the bromine reaction to a much greater extent than that of the chlorine reaction. The pertinent data is summarised in table 2.7.

Formic acid dissolves intercellular cement [Bradbury et al. (1965a, 1966, 1967)] and probably also removes at least some endocuticle material (see chapter 3). Dilute hydrochloric acid attacks endocuticle [Leach et al. (1964)]

TABLE 2.7. SUMMARY OF PRETREATMENTS

WHICH PREFERENTIALLY AFFECT THE HERBIG REACTION.

Treatment	Reaction time (secs.)	
	Chlorine	Bromine
Untreated	15	30-50
HCOOH, 70°C, 5 hours	17	15-25
0.01N HCl, 100°C, 20 hours	10	7-10
Phosphotungstic acid	18	100-150
Pronase digestion	15	16-20

and, judging from the enhanced separation of cuticle material following a partial hydrolysis pretreatment (section 2[E](i) and (ii)) this reagent also disrupts intercellular cement.

Phosphotungstic acid preferentially stains the endocuticle of Merino wool [see the electron micrographs of Bones and Sikorski (1967)] and forms deposits within the cell membrane complex of human hair, particularly after treatment with ethanol [Swift and Holmes (1965)]. Treatment of wool with proteolytic enzymes is considered to remove intercellular cement from the cell membrane complex [Rogers (1964)] and endocuticle from the scales (see Appendix).

Thus all the treatments listed in table 2.7 modify both intercellular cement and endocuticle, and change the rate of formation of Herbig sacs to a much greater extent than that of Allworden sacs. This indicates that these components are somehow involved in the reaction between bromine water and wool, which is very surprising since all other results implicate the outer layer of the exocuticle as the site of attack.

No explanation can be given for this effect!

The experiments and conclusions described in this chapter highlight two further questions regarding the resistant membranes of keratin fibres - viz. - (1) if epicuticle surrounds each cuticle cell, what is its origin and what is its relation to the whole-fibre cell membrane complex?, and (2) if epicuticle does not form a continuous external sheath, does it function to a measurable degree as a surface barrier to diffusion, and how permeable is this semi-permeable membrane?

These two questions form the subject of investigation in chapters 3 and 4, respectively.

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3. RESISTANT MEMBRANES, EPICUTICLE, AND THE CELL MEMBRANE COMPLEX.

[A] INTRODUCTION:

E. H. Mercer and G. E. Rogers, two of the main proponents of the continuous epicuticular sheath concept, have also proposed that epicuticle is a keratinised modification of the original cell membranes [Mercer (1957, 1961, 1965); Rogers (1959, 1964a)]. The results and discussions presented in chapter 2 have now removed the difficulty in reconciling a continuous epicuticle layer with an externally-discontinuous cell membrane; it remains only to demonstrate experimentally that epicuticle is, in fact, of cell membrane origin.

This chapter describes attempts to isolate and characterise whole-fibre cell membranes. Consideration is also given to the relation between epicuticle, cell membranes and the so-called 'cell membrane complex'.

The physical characteristics of epicuticle and (other) cell membranes precludes the use of mechanical methods for isolation, while their established chemical

resistance (see later) means that mild extraction techniques cannot be employed. Hence an effective isolation procedure will have to rely on selective dissolution of the fibre to leave a membranous residue.

The inter- and intra-molecular forces which occur in keratin fibres include -CO.NH- peptide bonds; -S.S- disulphide cross-links; hydrogen bonds (e.g. $\rangle C{=}O\ldots\ldots HN\langle$); salt links (e.g. $-COO^{-}\ldots\ldots{}^{+}H_3N-$); and van der Waals forces. Hydrophobic interactions, induced by the presence of water, also provide some degree of stabilisation [Leach (1959); Zahn (1964)]. Solvents which break the non-covalent types of bonds are capable of swelling keratin, but can extract only small quantities of material. Rupture of covalent bonds therefore appears to be necessary for any appreciable degree of dissolution of protein material from keratin fibres.

Factors which complicate attempts to isolate protein fractions from animal fibres by chemical means include (1) the complex histological structure of these fibres, and (2) the presence of two different types of protein i.e. disulphide cross-linked keratin material (approximately 90% of the fibre) and non-keratinous protein material (approximately 10% of the fibre) [Mercer (1961)].

[B] REVIEW OF PREVIOUS ATTEMPTS TO CHEMICALLY FRACTIONATE KERATIN FIBRES:

Almost every possible type of chemical attack has been employed in attempts to selectively remove components from wool and other protein fibres. Tables 3.1 and 3.2 contain a representative selection from the very large number of such attempts reported in the literature. They have been divided (somewhat arbitrarily) into non-specific and specific types of treatment.

Most dissolution methods involve attack on disulphide and/or peptide bonds, usually at a pH value removed from the iso-electric point of the protein (e.g. by the addition of alkali to confer a negative charge on ionisable acid groups, producing electrostatic repulsion between them). Incorporation of a hydrogen-bond breaking agent, such as urea, also helps to separate and untangle the molecular chains.

The literature prior to 1950 contains a confusing account of possible origins for the many preparations of resistant fractions of keratin fibres (see table 3.1). Many workers laid claim to discoveries of a (continuous) histological component situated between the cuticle and the cortex. These preparations were variously referred to as the 'sub-cutis'; the 'sub-cuticle membrane'; the 'intermediate layer'; the 'epidermis membrane'; and the 'cortex mantle' [see Mercer et al. (1949) and Mercer (1953)].

TABLE 3.1. PARTIAL DISSOLUTION OF KERATIN FIBRES BY 'NON-SPECIFIC' CHEMICAL ATTACK

Treatment	Residue	References
40% formaldehyde, 140°C- 2 hr. or 130°C- 3 hr.	5%	Lehmann (1941, 1943, 1944a,b).
Digestion by the common clothes moth (Tineola bisselliella).	'membranes'	Linderstrom-Lang and Duspiva (1935); Day (1951).
Supercontraction in phenol, 100°C- 2 hr., then digestion with trypsin.	5-10%	Elod and Zahn (1944, 1946).
Reduction with sod. thioglycollate, alkylation with ethyl bromide, then digestion with pepsin.	2-3% 'cuticular sheath'	Geiger et al. (1941); Geiger and Harris (1942); Zahn (1943); Hock and McMurdie (1943); Elod and Zahn (1947).
Conc. sulphuric acid, 0°C- 24 hr., reduction with sod. thioglycollate, then digestion with trypsin.	16%	Lustig et al. (1945).
Deamidation and hydrolysis with 0.05M cetyl sulphonic acid, 65°C - 6 days, then extraction with 0.001M sod. hydroxide.	30%	Lindley (1947).
Prolonged treatment with 1M sod. hydroxide at 20°C.	'transient membranes'	Zahn (1948).
0.3M sod. sulphide, 20°C - 4 weeks.	0.1%	Lindberg et al. (1948).
0.15M sod. sulphide, 40°C - 7 days.	0.7%	Schuringa et al.(1952a,b).
Formamide at 130°C, then digestion with pancreatin	5-10%	Zahn (1950).

(cont'd)

TABLE 3.1. (CONTINUED)

Treatment	Residue	References
1% papain, 0.1M sod. bisulphite, 4M urea, 50°C- 18 hr.	'cuticular sheaths'	Lennox (1952)
1M thioglycollic acid in phenol hydrate, 65°C - 10 days.	0.8%	Ross (1954)
Oxidation with 5% pot. dichromate in 1N sulphuric acid, 20°C- 18 hr., then extraction with 2N ammonia.	1.5 - 2%	Elliott and Roberts (1957)
47% sulphuric acid, 29°C - 5 days.	2%	Elliott et al. (1959).
98% sulphuric acid, 29°C - 4 days, then 10% sod. carbonate, 29°C - 30 min.	22%	Elliott et al. (1959).
0.05N pot. hydroxide, 100°C - 105 min.	20%	Elliott et al. (1959).
1M sod. borohydride, pH 11, 6M urea, 20°C - 1 hr.	9%	Gillespie (1959)
10M thioglycollic acid, 60°C - 24 hr.	'cell membranes'	Savige (1960)
Hydrochloric acid, pH 2, 100°C - 3 days	25%	Leach et al. (1964)

Closer examinations using the electron microscope and the phase-contrast optical microscope [e.g. Mercer et al. (1949)] revealed that these 'sub-cuticle' membranes consisted of epicuticle contaminated with various other remnants

from both cuticle and cortex. They did not, therefore, constitute separate histological components. Mercer (1950) coated fibres with gold prior to the isolation procedure; the isolated membranes were still coated with gold, demonstrating that the 'sub-cuticle' consisted, at least in part, of epicuticle. This indicated that the residue could not have originated from a layer beneath the cuticle.

The first attempt to fractionate wool by specific bond cleavage is probably that of Alexander and Earland (1950) - see table 3.2. After oxidation of the disulphide cross-links, these authors were able to dissolve 90% of the fibre in dilute ammonia solution; they claimed that the 10% residue was another demonstration of a sub-cuticle membrane.

Alexander and Earland's oxidation-extraction procedure excited considerable interest. The following workers showed that the residue consisted of epicuticle + cuticle cell remnants; cortical cell membranes; resistant fibrils; and nuclear remnants - Gralen et al. (1951); Mercer (1951, 1953); Peacock et al. (1951); Elliott and Manogue (1952); Manogue and Moss (1953); Manogue and Elliott (1953); Manogue et al. (1954); Fraser and Rogers (1955a); Rogers (1959).

Although their original interpretation was incorrect, Alexander and Earland must be given credit for pioneering the most widely-used method for selective extraction and

TABLE 3.2. PARTIAL DISSOLUTION OF KERATIN FIBRES BY 'SPECIFIC' ATTACK ON CYSTINE CROSS-LINKS.

Treatment	Residue	References
0.3M sod. bisulphite-10M urea, 40°C - 18 hr.	50%	Jones and Mecham (1943); Lees and Elsworth (1955).
1.6% peracetic acid, 20°C -25 hr., then extraction with 0.1 - 1.0M ammonia, 20°C for 12-24 hr.	7-10%	Alexander and Earland (1950).
1.6% peracetic acid, 20°C - 25 hr., then 0.1M ammonia, 20°C - 24 hr.	12%	Corfield et al. (1958).
Performic acid reagent (see later) 0°C - 16 hr. then extraction with solutions of various pH.	pH 9--46% pH10--40% pH11--20%	O'Donnell and Thompson (1959); Gillespie et al. (1960); Caldwell et al. (1966).
Oxidative sulphitolysis with 0.02M cupric ammonium hydroxide + 0.05M sod. carbonate + 8M urea, 20°C - 6 days.	10%	Swan (1957).
0.1M pot. thioglycollate pH 13.0, 50°C - 40 min.	26%	Gillespie and Lennox (1955).
0.2M pot. thioglycollate + 6M urea, pH 10.5, 20°C.	15-20%	Harrap and Gillespie (1963).
0.2M pot. thioglycollate + 10M urea, pH 11.0, 40°C - 2 hr.	9%	Gillespie (1964).
Reduction with mercapto-ethanol at pH 5, alkylation with iodoacetic acid at pH 8.5 and extraction at pH 11.	25%	Thompson and O'Donnell (1962).

characterisation of wool proteins (i.e. reduction or oxidation followed by alkaline extraction). Thus peracetic-oxidised wool can be separated into 3 main fractions - (1) α-keratose is precipitated when the ammonia-extract from peracetic-oxidised wool is brought to pH 4; (2) ɣ-keratose is the material remaining in solution after precipitation of α-keratose; (3) β-keratose is the fraction resistant to peracetic-ammonia treatment [Alexander et al. (1963), page 369].

It is currently accepted [see e.g., Crewther et al. (1965)] that the α, β and ɣ-keratoses are identifiable with the fibrillar, cell membrane and matrix components of the fibre, respectively.

What percentage of a wool fibre constitutes the resistant cell membrane material? If epicuticle is considered to be 30-50Å thick and to occur on both sides of a cuticle cell, then, for a fibre of 20μ diameter with a scale overlap of one-sixth, this membrane will contribute approximately 0.25% to the weight of the fibre. When allowance is made for the relative surface:volume ratios of cuticle cells and cortical cells; for the wrinkled surface of cortical cell membranes [Mercer (1953)]; and for the 9:1 ratio of cortex:cuticle, an estimate of 1-2% is obtained for the resistant cell membrane fraction.

Many of the treatments listed in table 3.1. leave a residue of 2% or less, but in every case extensive chemical degradation was necessary to achieve the resistant residue. Table 3.2 contains details of treatments which are more specific and less degradative, but in no case was a residue obtained which approximated to the weight expected for a 'pure' membrane fraction.

Because the membranes are resistant to chemical attack, they invariably remain after chemical dissolution of the bulk of the fibre, but always in admixture with other cellular remnants and/or in a chemically degraded state. Thus, Mercer (1953) states that most procedures which remove the keratin proper, leave a confusing mixture of resistant fragments from all histological regions; he also considers [Mercer (1965)] that Alexander and Earland's peracetic-ammonia treatment produces a relatively pure sample of membrane complex.

In the present work, several of the membrane-producing methods detailed in tables 3.1 and 3.2 were examined with a view to obtaining a pure preparation of resistant membranes, without excessive chemical breakdown.

[C] MATERIALS AND METHODS:

(i) Wool:

The ether-extracted and water-washed Merino 64's wool

sample described in section 2[C](i) was used for the studies on resistant membranes.

(ii) Reagents:

Chemicals and solvents were of A.R. grade where available. All solvents used in gravimetric experiments were distilled before use to ensure freedom from residues.

(iii) General Procedure for Membrane Estimation:

The following procedure was developed for recovery and quantitation of the residue remaining after a chemical extraction experiment - The residue was washed 3 times at the centrifuge with 98-100 % formic acid, layered onto 1:1:1-trichloroethane (density 1.35) and centrifuged to remove cellulosic and other heavy contaminants. It was then washed again with formic acid, freeze-dried, and conditioned to laboratory humidity overnight. The weight of residue was measured after final drying in an oven at 110°C for 1 hour or under vacuum (10^{-4}mm Hg) at 100°C for 1 hour.

(iv) Amino Acid Analyses:

Approximately 4 mgm of protein material was dried under vacuum, weighed, and hydrolysed (in vacuo) at 110°C for 24 hours with 1 ml of redistilled 6N hydrochloric acid. After removing the acid in a rotary evaporator, the residue was made to 5.0 ml with a solution of 12.5% sucrose in 0.1N hydrochloric acid.

A known volume of the solution of hydrolysed protein

was separated into its constituent amino acids using the Technicon Amino Acid Analyser system. The ion-exchange column was an 8% cross-linked resin of sulphonated polystyrene [Piez and Morris (1960)]. The eluent was reacted with ninhydrin and the optical density recorded at 440 mμ and 570 mμ.

Two columns were employed. Using a buffer flow rate sufficient to give 10-hour runs, plus appropriate programming and timing devices, duplicate analyses could be done in less than 24 hours.

Beckmann standard amino acid mixture was used to calibrate the analyser, while internal standards (taurine, norleucine and α-amino-β-guanidinopropionic acid) were included in each hydrolysate to enable correction for day-to-day variations in the system.

Citrulline and proline are normally eluted simultaneously from the column, so the method of Holy (1966), using the ratio of the areas under the 440 mμ and 570 mμ peaks and the nett height of the peaks, was used to estimate the proportions of these two amino acids.

(v) Determination of Ash Content:

The Australian Microanalytical Service, Melbourne, carried out the ash analyses by combustion at 800°C.

(vi) Test for Carbohydrates:

The Molisch test, using α-naphthol and sulphuric

acid [Vogel (1956)] was applied to membrane preparations, both before and after hydrolysis with 6N hydrochloric acid at 110°C for 1 hour.

(vii) Extraction of Lipid Material from Whole Wool:

Wool was given a preliminary extraction under anhydrous conditions with tert-butanol, to remove surface lipid-type contaminants [Anderson and Leeder (1965a,b)], then cut into approximately 0.2 cm lengths. After immersion in 98-100% formic acid at 50:1 liquor:wool ratio, the wool was 'emulsified' in a Polytron blending machine at 0°C for 1 hour, then shaken vigorously for 24 hours at 20°C. The formic acid solution was collected by centrifugation and the insoluble residue was shaken for 5 days at 20°C in fresh formic acid. The formic acid solution was again collected by centrifugation, and the total amount of material soluble in formic acid was recovered by freeze-drying.

The formic acid extract contained lipids, proteins and inorganic matter [Bradbury et al. (1965a)]. The lipid material was separated by extracting with 2:1 (v/v) chloroform-methanol under reflux for 2 hours, the mixture centrifuged, and the chloroform-methanol solution washed with water. The chloroform phase was evaporated to dryness and the (lipid) residue dried at 10^{-4} mm Hg and 20°C for 18 hours.

(viii) Microscopy:

Optical microscopy and photomicroscopy were as described in section 2[C](v).

[D] RESULTS AND DISCUSSION:

3[D] (i) Attempts to Isolate Whole-Fibre Resistant Membranes:

(a) Extraction of oxidised wool:

Alexander and Earland (1950), and many workers since, have isolated 'membranes' by treatment with peracetic acid followed by extraction in dilute ammonia; the weight of this β-keratose residue was always around 10% of the weight of wool. Blackburn and Lowther (1951) treated wool with per-formic acid in formic acid and found 40% dissolution (after 18 hours) due to the oxidation treatment alone. (Peracetic acid oxidation results in a slight weight increase). They concluded that formic acid dissolves oxidised protein. Further, the residue from performic acid oxidation was reported to be almost dissolved by dilute sodium carbonate or sodium hydroxide at room temperature.

Thompson and O'Donnell (1959) also obtained 40% dissolution of wool by performic-formic acid treatment at $0^{o}C$ for 24 hours. These workers nevertheless concluded that performic acid broke fewer peptide bonds than did peracetic acid.

Extraction of performic-oxidised wool with dilute (0.1 - 1.0 M) ammonia solutions gave residues of 20% [O'Donnell and Thompson (1959)], 18% [Caldwell et al. (1966)] and 12% [Asquith and Parkinson (1966)]. Asquith and Parkinson also obtained 13% of β-keratose from human hair.

In the present work, the performic acid reagent was prepared by adding 1 part (v/v) 30% hydrogen peroxide to 9 parts 98-100% formic acid and allowing the mixture to stand at 20°C for 2 hours [Thompson and O'Donnell (1959)]. Treatments were done at 250:1 liquor:wool ratio.

Initially, oxidations were carried out at 0°C for 24 hours. This gave 40% dissolution, in agreement with Blackburn and Lowther (1951) and Thompson and O'Donnell (1959); subsequent extraction with ammonia did not reduce this residue to less than 15% of the original weight of wool.

In another series of experiments, oxidation was allowed to proceed for 2 weeks at 20°C - this gave residue weights of around 5.6%. Subsequent extractions by gentle agitation at 20°C and 100:1 liquor:wool ratio in (1) 1.0 M ammonia, and (2) 8 M urea made to pH 10 with ammonia, gave the results listed in table 3.3, illustrated by figure 3.1.

The shapes of the curves in figure 3.1 show that a resistant fraction of the wool has been isolated. The membranous nature of the residue is shown in figure 3.2. Accordingly, it is proposed that oxidation of wool with

TABLE 3.3. EXTRACTION OF PERFORMIC-OXIDISED WOOL WITH 1 M AMMONIA OR 8 M UREA

Time of extraction	Residue (% of original weight of wool) after extraction with -	
	1 M ammonia	8 M urea, pH 10
0	5.6	5.6
6 hours	4.1	-
31 hours	2.1	-
3 days	1.5	3.5
1 week	1.35	1.55
2 weeks	1.25	-
6 weeks	1.05	1.30

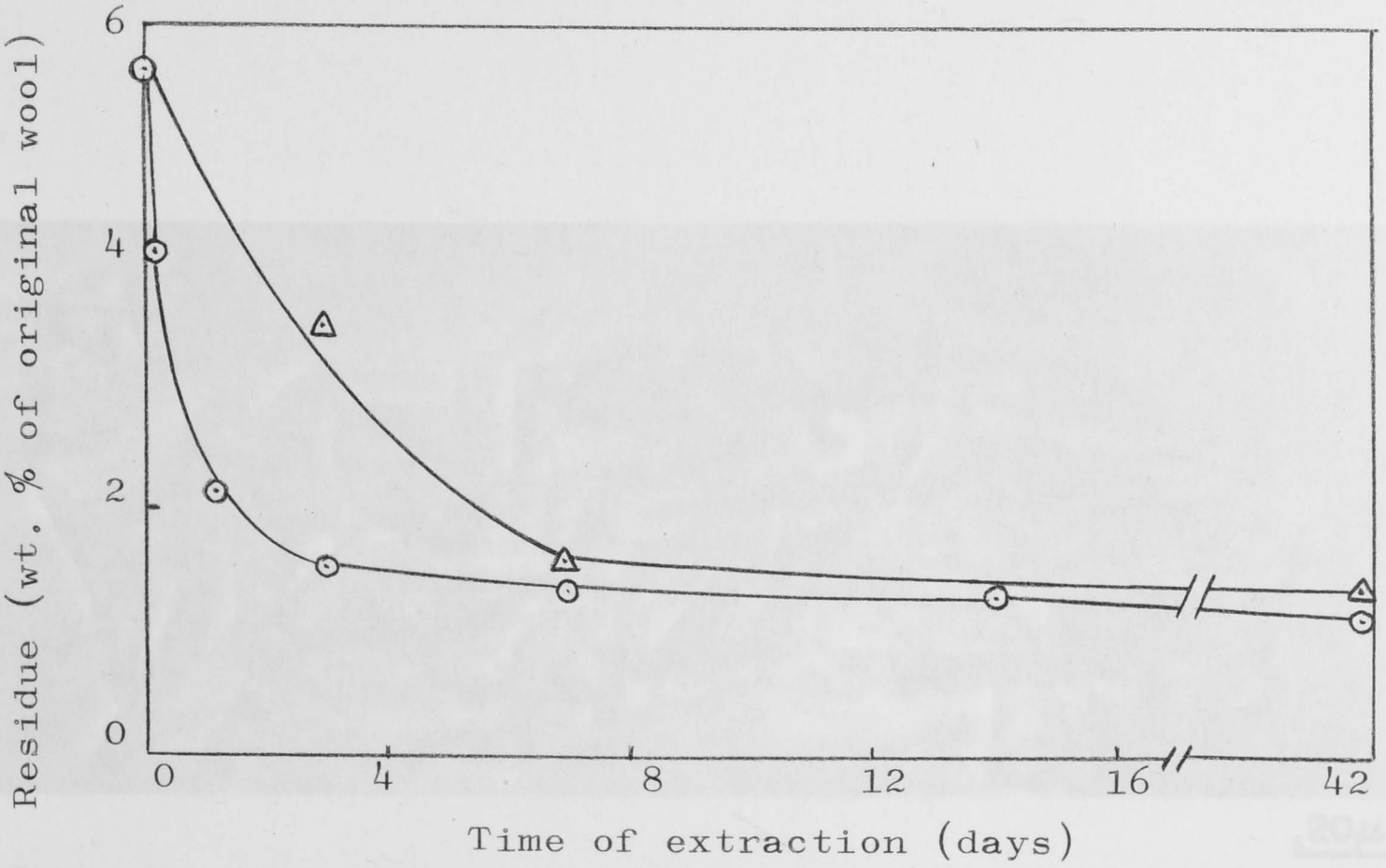

Figure 3.1. Extraction of Performic-Oxidised Wool with
(⊙) 1.0 M ammonium hydroxide
(△) 8 M urea at pH 10.

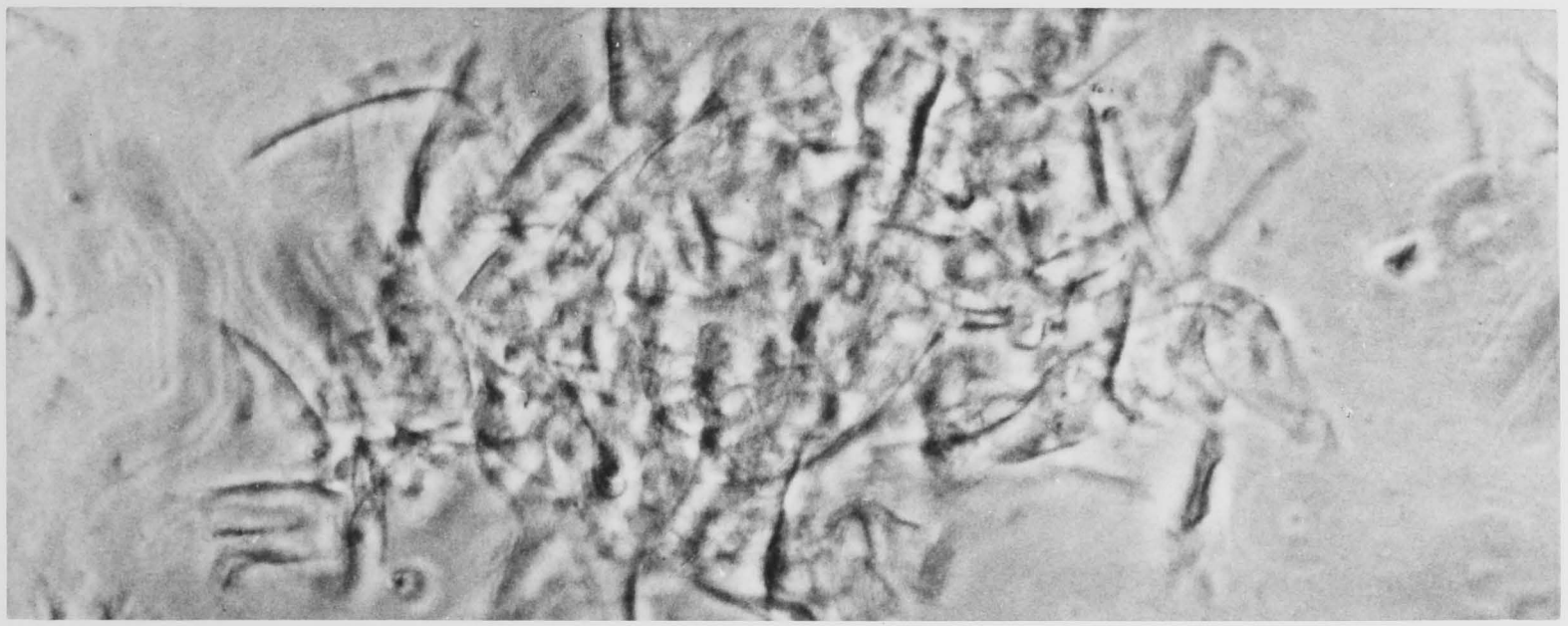

Figure 3.2. Residual Membranes after Performic-Ammonia Digestion of Merino Wool.

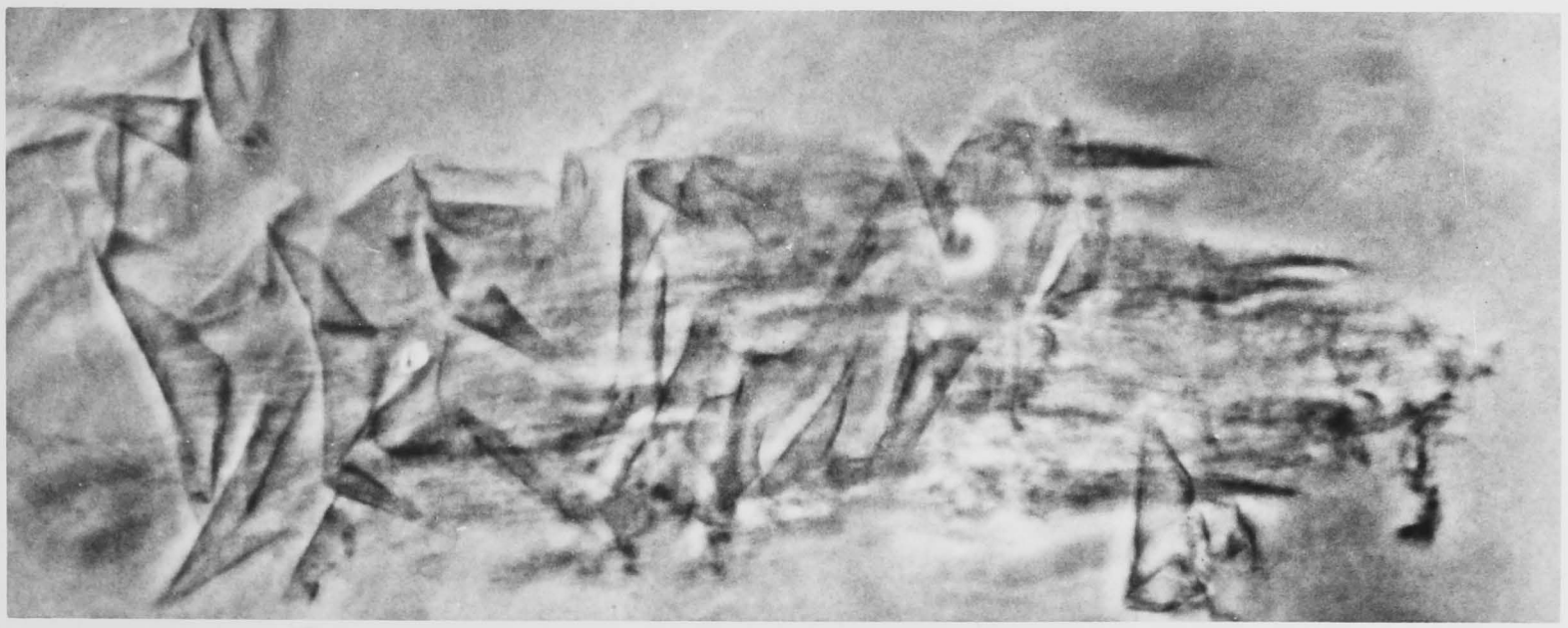

Figure 3.3. Residual Membranes after Digestion of Merino Wool with Dilute Potassium Hydroxide.

performic acid at 20°C for 2 weeks, followed by extraction with 1 M ammonia or 8 M urea at pH 10 for approximately 1 week, constitutes a method for isolation of the resistant cell membranes. The 1.5% residue approximates to that expected for these membranes. Analytical data and discussion of the results will be given in section 3[D](ii).

(b) Reduction with tributyl phosphine:

Following the success of oxidation-extraction for preparation of a resistant fraction, reduction-extraction experiments were carried out.

The disulphide groups of keratin fibres are quantitatively reduced under mild conditions with tributyl phosphine [Sweetman and Maclaren (1966) ; Maclaren and Sweetman (1966)]. The following treatments were applied.

0.5 gm wool was treated with 1 ml tributyl phosphine (i.e. 20 times the stoichiometric amount) in 100 ml 75% aqueous formamide, at 20°C for 24 hours. The reduced wool was then transferred to 100 ml 98-100% formic acid, shaken with a Vibromix agitator at 20°C for 4 hours, and finally shaken in the formic acid (on a laboratory shaker) at 20°C for 4 days. One such treatment gave a residue of 33%, 2 treatments gave 19% and 3 successive treatments produced a residue of 15%. Aftertreatment of the 15% residue with 1 M ammonia at 20°C for 4 days reduced this figure to 13%.

Similar experiments on wool pretreated with pronase

(see Appendix) surprisingly gave a minimum residue of 25%. The enzyme pretreatment had removed 12% of material, so the final residue was expected to be decreased rather than increased. Lanthionine formation during the pronase digestion (4.5 weeks at 37°C and pH 8) may have stabilised the wool structure.

Disappointing results were also obtained when the reduction was carried out in 20% aqueous propanol and the reduced disulphide groups blocked with methyl iodide, prior to extraction with formic acid or ammonia.

In each case, formation of small amounts of lanthionine during the tributyl phosphine treatments [Jenkins and Wolfram (1963); DeDeurwaeder et al. (1964); Sweetman and Maclaren (1966)] may have prevented extensive dissolution of the fibres. The charge and polarity of the half-cystine residues will undoubtably also affect the degree of extraction, e.g. if a blocking agent of similar charge and solubility to the $-SO_3^-$ groups present in oxidised wool, could be applied to reduced wool, perhaps greater total extraction could be achieved. The non-degradative nature of tributyl phosphine reductions and the nearly-quantitative -S.S- cleavage emphasises the potential of this reagent for protein fractionation.

(c) Digestions with dilute potassium hydroxide:

Treatment with 0.4 M potassium hydroxide at 200:1

liquor:wool ratio and 20°C gave the following results - after 1 week, 2.3% residue (see figure 3.3); after 2 weeks, 0.1% residue; after 3 weeks, total dissolution. The membranous nature of the residue after 1 week is shown in figure 3.3, but the severe degradation inherent in the treatment is shown by the rapid total dissolution of the fibre. Zahn (1948) observed that dilute sodium hydroxide dissolved the scales and cortical cells first, the cortical cell membranes dissolving at a slower rate.

(d) Digestions with sulphuric acid:

Dissolution of wool in strong sulphuric acid followed a similar pattern to that in dilute potassium hydroxide. Treatment with 15 N sulphuric acid at 200:1 liquor:wool ratio and 20°C gave 1.2% residue after 1 week, 0.4% residue after 2 weeks and total dissolution after 3 weeks.

Elliott et al. (1959) obtained a 'membrane' preparation which represented 2% of the wool after 5 days in 17 N sulphuric acid. The dissolution followed a first-order reaction rate, indicating non-specific hydrolytic attack on the fibre.

(e) Digestion with sodium sulphide:

Lindberg et al. (1948) isolated membranes by prolonged immersion of wool in dilute sodium sulphide solution; electron microscopic examination showed these membranes to be similar to epicuticle produced by shaking

chlorine-heated fibres in water. Lindberg et al. therefore concluded that sodium sulphide digestion represented an alternative method for isolation of epicuticle.

In the present work, wool was treated with 0.2 M aqueous sodium sulphide, at 100:1 liquor:wool ratio and 20°C for 30 weeks. The residue was washed at the centrifuge with sodium sulphide solution, and immersed in fresh 0.2 M sodium sulphide for a further 2 weeks. The standard quantitation technique yielded a residue representing 1.2% of the weight of wool.

A photomicrograph of the sodium sulphide residue was presented in figure 2.22, page 47a. Comparative microscopic examination under phase contrast and under bright field illumination showed that the membranes were much thicker than those obtained by performic-ammonia treatment (figure 3.2) or digestion in dilute alkali (figure 3.3); cortical cell membranes did not appear to be present; the preparation therefore resembled that of epicuticle (see figure 2.16, page 44a).

Could the sodium sulphide residue represent a fraction other than whole-fibre cell membranes? The weight of residue was more than 0.25% expected for epicuticle. King and Bradbury (1967) could not dissolve more than 87% of a cuticle preparation by prolonged treatment with sodium sulphide. This 13% cuticle-residue would represent 1.3% of

the weight of whole fibre provided no other remnants are present - a similar figure to that obtained above. Schuringa et al. (1952a) found that a sodium sulphide residue was resistant to trypsin, while a Herbig membrane preparation readily dissolved, suggesting that the former residue was not of epicuticular origin.

A possible explanation is that the sodium sulphide residue originates from the 'a' layer of the exocuticle. This layer represents 1-2% by weight of the wool (see table 1.2, page 8); it is considered to be more resistant to enzymes and keratinolytic reagents than the other components of the cuticle [see, e.g. Dobb et al. (1961) and references therein]. Conversion of cystine to lanthionine by the alkaline sodium sulphide solution could preferentially stabilise the 'a' layer because of its very high disulphide content. Thus Schuringa et al. (1952a) detected lanthionine in their sodium sulphide residue, and proposed that the presence of these cross-links conferred the observed chemical resistance.

Amino acid analyses and other tests are needed to decide whether the sodium sulphide residue originates from cell membranes or the 'a' layer of the exocuticle. At this stage it will be assumed that digestion of wool in sodium sulphide is not a method for producing whole-fibre cell membranes.

It is interesting to note that egg-shell membranes

(ovokeratin) also leave a 13% residue when digested with sodium sulphide [Jones and Mecham (1943)].

(f) Digestion with concentrated thioglycollic acid:

Savige (1960) found "a small amount of residue consisting of cell membranes" when wool was treated with 10 M thioglycollic acid - 0.5 M acetic acid at 60°C for 24 hours. This was confirmed during the present work, but again the membranes were only a transient stage in total dissolution of the fibres.

(g) Extraction with hot formic acid:

Immersion of wool in formic acid at 20°C results in slow but continuous dissolution of protein material, the first fractions of which have a very low cystine content [Bradbury et al. (1965a); King (1967)]. This suggested the possibilities that (1) by increasing the rate of dissolution (e.g. by increasing the temperature) a cell membrane residue might be obtainable, and (2) formic acid might represent a solvent for preferential extraction of the non-keratinous fraction of keratin fibres. However, figure 3.4 shows that the graph of percent dissolution versus time of extraction with formic acid at 100°C, does not form a plateau at around the 10% value expected for the non-keratinous content of wool.

When wool was heated in a sealed evacuated glass tube at 110°C for 24 hours in the presence of formic acid,

total dissolution occurred. (This procedure is not recommended - when an attempt was made to open the tube after the 110°C treatment, a violent explosion occurred. Apparently gaseous decomposition products from wool and/or formic acid had built up a high pressure in the sealed tube).

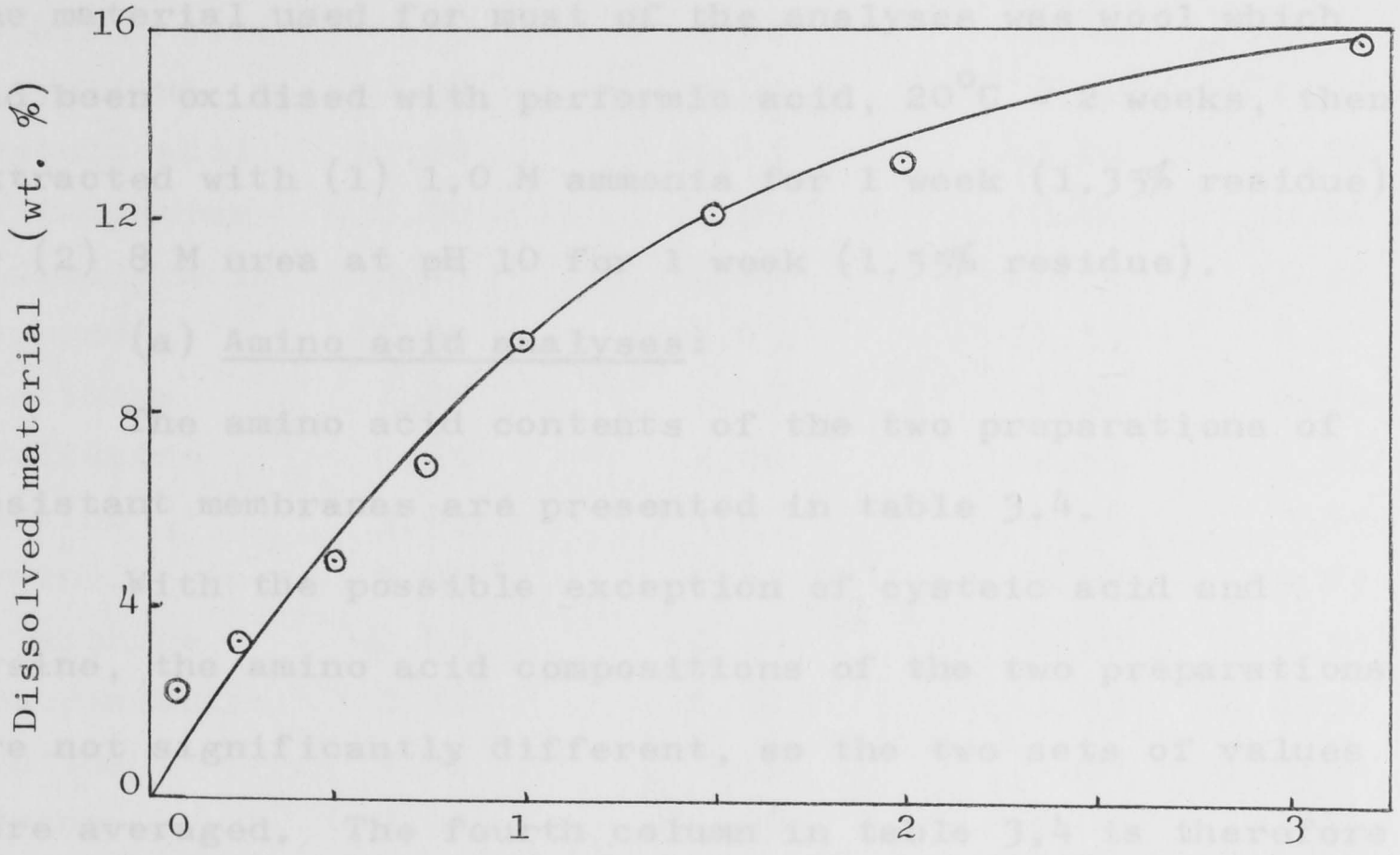

Figure 3.4. Rate of Dissolution of Merino Wool by Formic Acid at 100°C.

3[D](ii) Characterisation of the Resistant Membranes:

Several of the treatments described in the preceding section resulted in membranous residues of around the 1-2% expected for the resistant cell membranes, but only the performic-ammonia and performic-urea residues were stable to prolonged digestion in the extracting medium. Analyses were therefore performed only on these two preparations. The material used for most of the analyses was wool which had been oxidised with performic acid, 20°C - 2 weeks, then extracted with (1) 1.0 M ammonia for 1 week (1.35% residue), or (2) 8 M urea at pH 10 for 1 week (1.55% residue).

(a) Amino acid analyses:

The amino acid contents of the two preparations of resistant membranes are presented in table 3.4.

With the possible exception of cysteic acid and lysine, the amino acid compositions of the two preparations are not significantly different, so the two sets of values were averaged. The fourth column in table 3.4 is therefore taken to represent the amino acid analysis of the resistant cell membranes of Merino wool. For purposes of comparison, column 5 of table 3.4 lists the amino acid content of unmodified Merino wool.

(b) Ash content:

Ash contents of the performic-ammonia and performic-urea residues were 6.9% and 5.6% respectively. Agreement

TABLE 3.4.

AMINO ACID ANALYSES (MOLE %) OF RESISTANT MEMBRANES FROM MERINO WOOL.

Amino Acid	Performic-ammonia Residue	Performic-urea Residue	Average	Whole Wool[a]
Alanine	6.90	6.09	6.50	5.31
Arginine	4.26	4.12	4.19	6.80
Aspartic acid	5.79	5.02	5.40	6.34
Citrulline	0.00	0.00	0.00	0.07
Cysteic acid	11.60	14.43	13.02	0.08
Half-cystine	0.00	0.00	0.00	10.44
Glutamic acid	10.43	10.25	10.34	11.86
Glycine	13.90	14.43	14.17	8.57
Histidine	1.29	1.37	1.33	0.93
Isoleucine	2.65	2.46	2.55	3.11
Leucine	5.08	4.67	4.88	7.65
Lysine	9.00	7.69	8.35	3.05
Methionine	0.00	0.00	0.00	0.50
Phenylalanine	1.53	1.52	1.53	2.90
Proline	7.01	7.13	7.07	5.90
Serine	9.99	10.08	10.04	10.21
Threonine	5.89	5.57	5.73	6.47
Tyrosine	0.00	0.00	0.00	3.95
Valine	4.66	5.16	4.91	5.50
Anhydroamino Acid Recovery	76.7%	86.3%	81.5%	96%

[a] from Bradbury et al, (1965b).

between the two values is again good. The slightly lower weight of the performic-ammonia membranes would have resulted in the inorganic (contaminants?) forming a greater proportion of this preparation.

(c) Tests for carbohydrates:

Negative results were obtained when the Molisch test was applied both before and after hydrolysis of the membrane preparations with hydrochloric acid.

The analytical data presented above allows considerations of the general properties of resistant cell membranes and their relationship to epicuticle and the cell membrane complex.

The analyses show that these resistant membranes contain approximately 82% protein and 6% inorganic matter. Thus 12% of the material is unaccounted for. Part of this 12% is due to losses during hydrolysis. King (1967) reports that 6 N hydrochloric acid at 110°C for 24 hours results in losses of 9% serine, 3% cystine, 6% threonine and 6% tyrosine. In accordance with common practice in this department, these losses were not corrected for.

Slight errors in weighing, in the analysis technique, and in quantitation of the chromatograms probably also contributed to the discrepancy. It is also possible that

substances other than protein or inorganic matter were present in the preparations.

Regarding the amino acid analyses in table 3.4, the absence of tyrosine and methionine does not mean that these amino acids are not present in the unmodified membranes. Performic acid is known to attack tyrosine and methionine [Toennies and Homiller (1942); Blackburn and Lowther(1951)].

Note the complete absence of cystine, which is presumably quantitatively oxidised to cysteic acid by the performic acid [Thompson and O'Donnell (1959)].

The proportions of amino acids in the resistant membranes is different to those in whole wool (table 3.4). However, there is nothing about the analyses to explain the chemical inertness of this material. Many workers have predicted that a very high cystine content may be responsible for this resistance, but the present results do not support this theory. The cystine content of the membranes is only 25% greater than that of whole wool, and is actually less than that of Merino cuticle (table 1.3). Furthermore, the oxidised membranes are more resistant to dissolution by formic acid and ammonia than the rest of the fibre.

In his interesting review on cell membranes, Mercer (1965) states that they are resistant to a wide range of keratinolytic reagents, but are dissolved by trypsin and pepsin. Because of the keratin-like amino acid analysis

of the membranes (table 3.4), Mercer's statement did not seem likely, so the resistance of the membranes was checked by applying enzyme treatments both before and after the performic-ammonia procedure. When trypsin or pronase solutions (at pH 8) were applied to isolated membranes, they dissolved within a few minutes, but when a 3-week pronase pretreatment was applied (12% of the wool dissolved - see Appendix) a membranous residue similar to the 'normal' performic-ammonia residue, was obtained. Thus the resistant membranes behave in a similar manner to normal keratin, in that they are resistant to enzymatic attack provided that the disulphide cross-links are intact.

Table 3.5 compares the amino acid analysis for the membranes isolated during the present work, with those for 'membranes' prepared by digestions in sulphuric acid [Elliott et al. (1959)], peracetic-ammonia [Corfield et al. (1958)] and a milder performic-ammonia treatment [Gillespie et al. (1960)].

The amino acid analysis for Elliott et al.'s sulphuric acid residue shows several major differences from that of membranes isolated in the present work. This is probably due to the different nature of the reagents used and to the extensive hydrolysis which would have occurred during the sulphuric acid treatment.

The two β-keratose preparations listed in columns

TABLE 3.5.

AMINO ACID ANALYSES (MOLE %) OF RESISTANT FRACTIONS FROM MERINO WOOL.

Amino Acid	Resistant Membranes (present work)	Sulphuric Acid Residue[a]	Peracetic-ammonia[b] Residue	Performic-ammonia[c] Residue
Alanine	6.50	5.32	6.78	6.63
Arginine	4.19	6.16	6.22	9.51
Aspartic acid	5.40	2.82	6.66	6.24
Cysteic acid	13.02	0.00	6.16	9.75
Half-cystine	0.00	22.40	0.00	0.00
Glutamic acid	10.34	11.17	10.24	11.97
Glycine	14.17	3.69	9.16	7.60
Histidine	1.33	0.68	1.23	1.59
Isoleucine	2.55	2.61	4.00	3.01
Leucine	4.88	6.00	8.65	7.92
Lysine	8.35	2.35	4.52	5.93
Methionine	0.00	1.18	0.00	0.00
Phenylalanine	1.53	1.39	3.29	2.19
Proline	7.07	12.78	6.40	6.74
Serine	10.04	10.03	11.20	10.70
Threonine	5.73	6.12	6.16	4.90
Tyrosine	0.00	1.94	3.15	1.77
Valine	4.91	3.35	6.18	3.55
Anhydroamino Acid Recovery	81.5%	90.6%	98.2%	-
Weight % of wool	1.5%	2%	12%	20%

[a] from Elliott et al. (1959)

[b] from Corfield et al. (1958)

[c] from Gillespie et al. (1960).

4 and 5 of table 3.5 consist of more than the 1-2% expected for 'pure' resistant cell membranes, and have been shown to include much other material (see section 3[B]). In particular, the low cystine content of the peracetic-ammonia residue suggests the presence of large amounts of non-keratin material.

3[D](iii) The Origin of Epicuticle:

The possibility that epicuticle originates from the resistant cell membranes can now be checked, by comparison of the amino acid composition of epicuticle [from King and Bradbury (1967)] and whole-fibre cell membranes isolated during the present work. Table 3.6 contains the pertinent data.

The analyses show differences in alanine, lysine and proline; tyrosine was destroyed by the performic acid so cannot be considered; the cystine + cysteic acid values agree well, as do those for serine + threonine - the optical density plots for the latter two amino acids tend to overlap on the chromatograms.

Extensive chemical treatments were required to isolate both membrane preparations listed in table 3.6 (chlorine treatment in one case, and performic-formic-ammonia or performic-formic-8 M urea in the other case). The agreement between the two sets of analyses is considered

TABLE 3.6.

COMPARISON OF THE AMINO ACID ANALYSES (MOLE %) OF EPICUTICLE AND WHOLE-FIBRE CELL MEMBRANES.

Amino Acid	Whole-fibre Resistant Membranes	Epicuticle[a]
Alanine	6.50	4.66
Arginine	4.19	4.31
Aspartic acid	5.40	5.89
Citrulline	0.00	0.90
Cysteic acid	13.02	11.88
Half-cystine	0.00	0.33
Glutamic acid	10.34	10.76
Glycine	14.17	15.50
Histidine	1.33	1.04
Isoleucine	2.55	2.54
Leucine	4.88	5.51
Lysine	8.35	4.88
Methionine	0.00	0.03
Phenylalanine	1.53	1.86
Proline	7.07	5.85
Serine	10.04	13.78
Threonine	5.73	3.62
Tyrosine	0.00	2.08
Valine	4.91	5.77

[a] from King and Bradbury (1967).

to be sufficient to state that epicuticle is chemically similar to the resistant whole-fibre cell membranes.

An estimate of the thickness of the whole-fibre membranes was made by electron-microscopic examination,* of a membrane preparation after shadowing with platinum at an angle of 7.5^{o}. From the width of 'shadow' and the geometry of the system, an approximate value of 30Å was obtained. This agrees with the figure of $32 \pm 10\text{Å}$ obtained by King and Bradbury (1967), but the resolution of the electron microscope used was not sufficient to give an accurate thickness measurement.

Further proof of the equivalence of epicuticle and whole-fibre resistant membranes is provided by their common resistance to acids, alkalies, oxidising agents, reducing agents and enzymes. Tables 2.2 and 2.4 contain the data for epicuticle, while tables 3.1 and 3.2, and the results presented above demonstrate the resistance of the whole-fibre membranes.

It is notable that Blackburn and Lowther (1951) obtained performic-ammonia membranes from descaled fibres. This demonstrates that at least part of the membranes obtained in the present work are from the cortex of the

* [The author is indebted to Dr. E. G. Brittain of the Botany Department, Australian National University, for help with the electron-microscopic examinations.]

fibre, and are not just 'thickened epicuticle' as suggested for many membrane preparations [Mercer et al. (1949); Mercer (1953)].

To summarise, the results and discussion presented above confirm that epicuticle is not an extracellular layer, but consists of that part of the keratinised cell membrane residue which occurs on the outer surface of the fibre.

3[D](iv) The Cell Membrane Complex:

The so-called 'cell membrane complex' exists between all cells in assemblies of keratinised tissues. A typical electron micrograph of the area between two cells in a wool fibre is shown in figure 3.5. Structures which stain in this manner with heavy metals have been observed in cross-sections of wool fibres [Rogers (1959); Bradbury et al. (1965a)]; human hair [Birbeck and Mercer (1957a); Swift and Holmes (1965)]; feather rachis [Filshie and Rogers (1962)]; the inner root sheath of sheep-skin follicles [Rogers (1964a)]; and the outer- and inner-root sheaths of human skin follicles [Birbeck and Mercer (1957a)].

Figure 3.7 shows schematically the relation between the cell membrane complex as seen under the electron microscope, and the picture built up from considerations of keratinisation and differentiation of the various components during hair growth [see, e.g. Birbeck and Mercer (1957a,b); Sjostrand (1962); Swift and Holmes (1965)].

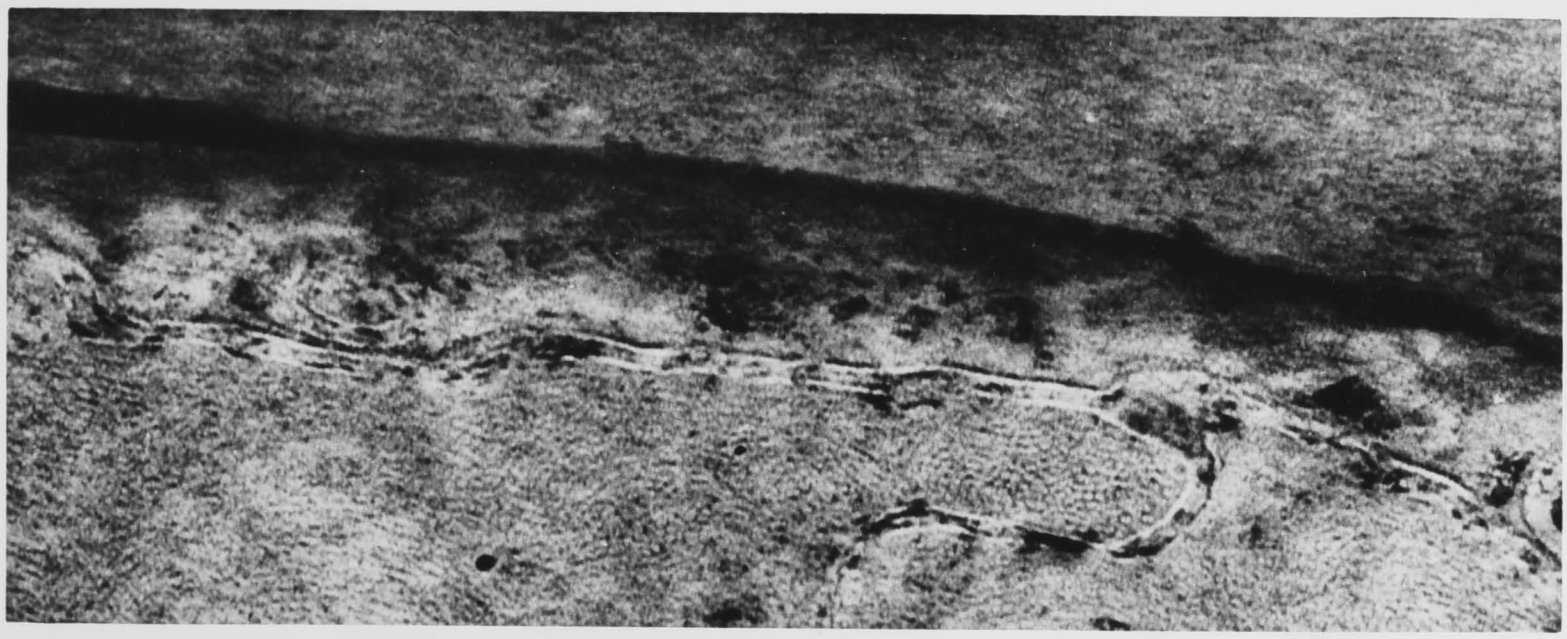

Figure 3.5. Cross-section of a Merino Fibre, Stained by the Thioglycollic Acid-Osmium Tetroxide Method to Show the Cell Membrane Complex (from Bradbury et al.(1965a)).

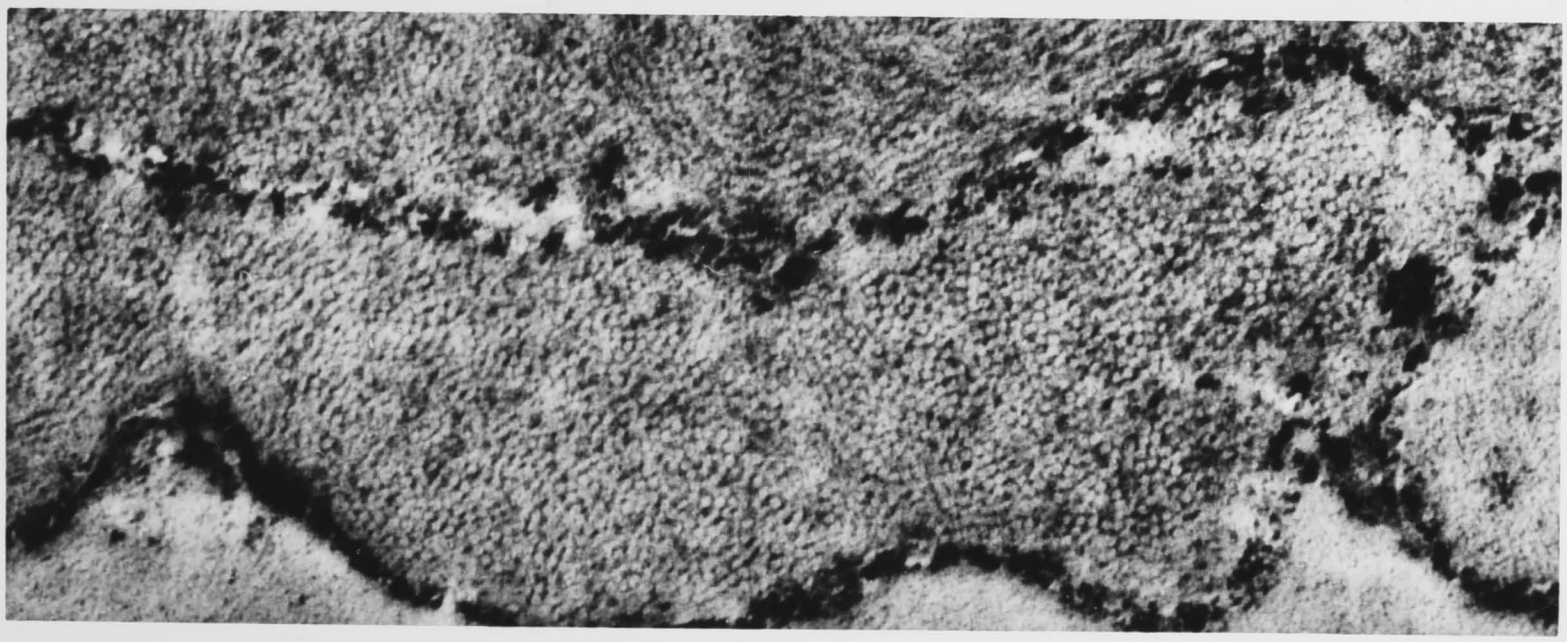

Figure 3.6. Osmium-stained Cross-section of a Merino Fibre, Preatreated with Dichloroacetic Acid at 20°C for 12 hours (from Bradbury et al.(1965a)).

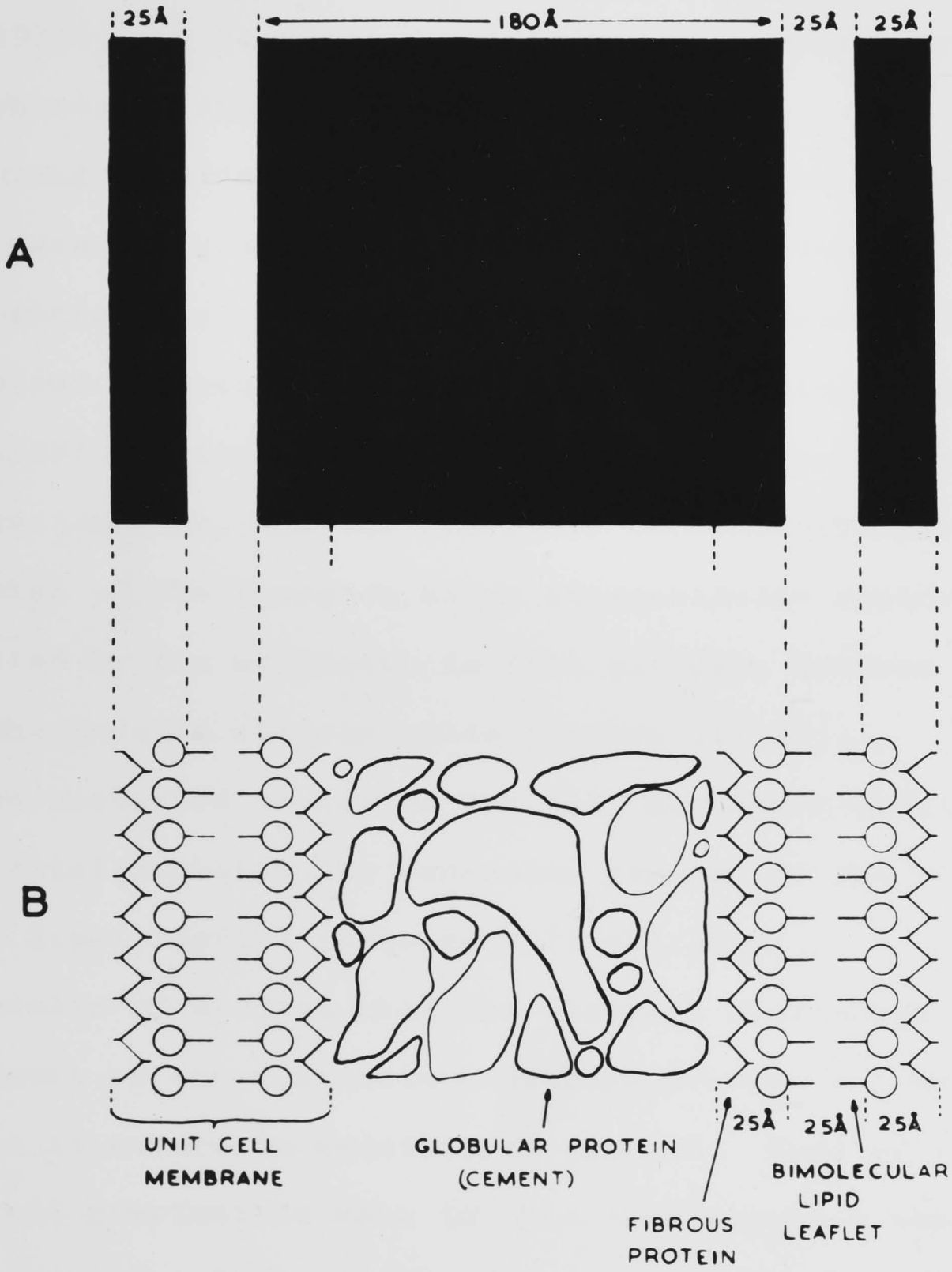

Figure 3.7. Schematic Representation of the Relation Between (A) the Structure Seen Under the Electron Microscope, and (B) the Cell Membrane Complex, According to Swift and Holmes (1965).

The structure of the 'unit cell membrane' as it occurs in living systems, was originally proposed by Danielli and Davson (1935), and is still applicable as a general working model [Robertson (1966)].

During keratinisation in the follicle, the intercellular material gradually differentiates between the unit cell membranes of adjacent cells [Birbeck and Mercer (1957b)]. This material therefore constitutes an extracellular deposit. Rogers (1959) has termed the (osmophilic) intercellular cement the δ-region, and the unstained sections the β-region. The function of the δ-region as an intercellular cement is demonstrated by its expansion to fill out gaps between cells, e.g. at the ends of cuticle cells [Rogers (1959)].

The unstained layers of the cell membranes consist of lipid material; probably the non-polar regions of the bi-molecular lipid leaflet [Robertson (1959, 1966)].

Results of studies with the electron microscope have provided most of the available knowledge of the cell membrane complex as it occurs in keratinised tissues. Some of the chemical and gravimetric data in this thesis have a bearing on the structure and chemistry of the cell membrane complex; current knowledge of the various layers of this complex will be briefly considered in terms of these results -

(a) Resistant cell membranes:

The inner fibrous protein layer of the original unit

cell membrane is tentatively proposed as the source of the resistant whole-fibre membranes isolated from keratinised tissues (this includes, of course, epicuticle).

The total weight of this resistant protein layer was shown to be 1.5% of the weight of wool, and the thickness was assumed to be the same as epicuticle i.e. 32 $\pm$ 10Å.

But there are <u>two</u> protein layers in the unit cell membrane (see figure 3.7). The fate of the outer protein layer is not known. Robertson (1966) considers this outer layer to have different chemical properties to the inner layer, so perhaps it forms part of the intercellular cement and/or is dissolved by the treatments used to isolate the resistant membranes. An alternative explanation is that the isolated resistant membranes consist of <u>both</u> protein layers. The second possibility receives support from the work of Rogers (1964a) - he removed the intercellular cement from inner root sheath cells by extraction with urea, thereby exposing a complete unit cell membrane structure around each cell. The protein layers of the unit cell membranes cannot normally be seen because they are indistinguishable from the equally-stained proteins of the cells and the δ-layer.

A closer definition of the relation between the resistant membranes of keratinised structures and the protein layers of the original cell membranes must await

the acquisition of new and more precise data on the chemical and physical structure of the unit cell membrane in living systems, and of the changes which occur during keratinisation.

(b) Intercellular cement:

Burgess (1934) was probably the first to suggest that the individual cells of keratin fibres were held together by an intercellular cement. The amount present in keratin fibres is not known, but several authors have published analytical data for proteins which they assume to originate in this cementing layer.

The thickness of intercellular cement is generally agreed to be close to 150Å [Birbeck and Mercer (1957b); Rogers (1959); Filshie and Rogers (1962); Swift and Holmes (1965)]. Using this figure, and the value of 1.5% by weight for resistant membranes, an estimate of the amount of intercellular cement present can be made. There will be 2 resistant membranes approximately 32Å wide associated with each 150Å layer of intercellular cement. Thus, assuming equal densities, the weight of intercellular cement present in Merino wool will be $1.5 \times \frac{150}{64} = 3.5\%$. Since the thickness of the intercellular layer increases to fill out gaps in the structure [Rogers (1959)] this figure of 3.5% is probably a minimum value, although the errors and assumptions involved do not allow a closer estimate to be made at this time.

Figures 3.5 and 3.6 showed the staining behaviour of the cell membrane complex before and after extraction with dichloroacetic acid. The same effect is obtained by extraction with formic acid [Bradbury et al. (1965a)], showing that these solvents greatly modify the cell membrane structure. The 1% of material extracted by formic acid in 1 hour at 20°C [Bradbury et al. (1965a)] consisted mostly of lipids, but a protein content of approximately 0.3% (on weight of wool) was present in the extract.

Zahn and Biela (1968) obtained 0.4% of protein material by extracting wool with 50% aqueous formic acid at 20°C for 1 hour, while Blackburn and Lowther (1951) extracted 0.45% protein with 100% formic acid at 20°C for 48 hours. The separation of cuticle and cortical cells after treatment of wool with formic acid demonstrates that this solvent modifies the cementing action of the intercellular material. Thus the protein extracted by formic acid is considered to come from the cell membrane complex. [Bradbury et al. (1965a, 1966, 1967), Bradbury and King (1967)].

DeDeurwaerder et al. (1964) extracted 2-3% of protein material from wool with diethylaminomethyl phosphine in formamide. Examination of the extracted fibres under the electron microscope showed that most of the intercellular cement had been removed, so an estimate of the weight of

intercellular cement from DeDeurwaerder et al's. results agrees well with the present estimate. The material extracted by Bradbury et al. (1965a) and by Zahn and Biela (1968) therefore represent only small fractions of this cement.

The analytical results obtained by DeDeurwaerder et al. [published by Andrews et al. (1966)] are compared with the results of Bradbury et al. (1965a) and Zahn and Biela (1968), in table 3.7. The amino acid analysis for untreated wool is included for comparative purposes.

Considering the different extractive techniques employed and the difference in amount of material extracted, the analysis by DeDeurwaerder et al. is surprisingly similar to that by Zahn and Biela. All three analyses possess the common features of low (cystine + cysteic acid) and high tyrosine, phenylalanine and glycine contents. Both DeDeurwaerder et al. and Zahn and Biela consider these features to be characteristic of intercellular cementing material. Zahn and Biela have tabulated published analytical data for a variety of readily-extracted protein fractions which exhibit these common features. Since the intercellular cement represents approximately 3.5% of the weight of wool, and since this component is very readily dissolved by a wide range of protein reagents (see table 3.8), it is not unreasonable to expect this cement to occur in many protein fractions from wool.

TABLE 3.7.

AMINO ACID ANALYSES (MOLE %) OF PROTEIN FRACTIONS FROM INTERCELLULAR CEMENT

Amino Acid	Phosphine Extract[a]	50% Formic Acid Extract[b]	100% Formic Acid Extract[c]	Whole Wool[d]
Alanine	2.63	2.15	6.23	5.31
Arginine	4.29	4.18	6.16	6.80
Aspartic acid	3.76	3.37	7.28	6.34
Citrulline	0.00	0.00	0.37	0.07
Cysteic acid	2.81	0.00	0.12	0.08
Half-cystine	0.74	0.80	2.07	10.44
Glutamic acid	0.91	2.91	10.50	11.86
Glycine	29.78	33.68	14.39	8.57
Histidine	0.49	0.33	1.74	0.93
Isoleucine	0.36	0.97	3.91	3.11
Leucine	4.95	7.94	8.21	7.65
Lysine	0.18	0.94	4.66	3.05
Methionine	0.02	0.00	1.20	0.50
Phenylalanine	9.38	5.76	4.33	2.90
Proline	5.83	4.13	4.03	5.90
Serine	13.44	12.63	8.33	10.21
Threonine	3.44	1.92	4.87	6.47
Tyrosine	14.68	16.44	6.20	3.95
Valine	2.32	2.02	5.47	5.50
Weight % of wool	2-3%	0.4%	0.3%	100%

[a] from DeDeurwaerder et al. (1964)

[b] from Zahn and Biela (1968)

[c] from Bradbury et al. (1965a)

[d] from Bradbury et al. (1965b)

The above considerations have helped to clarify the confusion which exists in the literature, between the very labile intercellular cement and the very resistant cell membrane material. The ready separation of cuticle and cortical cells when wool fibres are treated with enzymes [Mercer (1953); Manogue et al. (1954); Golden et al. (1955)] demonstrates that the intercellular cement is soluble in enzymes, while it was shown in section 3[D](ii) that the resistant membranes are not dissolved by enzymes.

TABLE 3.8.

CHEMICAL RESISTANCE OF PROTEINS FROM THE CELL MEMBRANE COMPLEX.

Reagent	Resistant Membranes	Intercellular Cement	Intracellular Keratin
Proteolytic enzymes	insoluble	soluble	insoluble
Formic acid	insoluble	partly soluble	resistant
Strong acids	resistant	soluble	soluble
Strong alkalies	resistant	soluble	soluble
Performic-alkali	insoluble	soluble	soluble
Reducing agents - pH 10-12	insoluble	soluble	mostly soluble
Sodium sulphide	soluble (?)	soluble	mostly soluble.

Electron microscopy by Rogers (1959) showed that the intercellular cement was soluble in performic-ammonia - again a difference in chemical reactivity from the resistant membranes.

The above information, together with other evidence collected from the body of this thesis and from the literature, is summarised in table 3.8, and compared with the chemical resistance of the keratin which constitutes the bulk of a wool fibre.

(c) Lipid content of the cell membrane complex:

The chloroform-soluble material from the exhaustive formic acid extraction detailed in section 3[C](vii) constituted 0.82% of the weight of wool. The 0.13% ash found in formic acid extracts [Bradbury et al. (1965a)] probably consists of alkali metal salts and phosphorus salts from the lipid layers. This gives a figure of approximately 1% for the lipid content of wool.

The weight of resistant membranes is 1.5%, so the value of 1% for lipid content indicates that the lipid layer of the unit cell membrane is approximately 20Å thick, assuming a resistant membrane thickness of 30Å. This thickness estimate increases to around 25Å when allowance is made for the difference in densities of keratin (1.3) and cholesterol-type lipids (1.0). A thickness of 25Å for the bimolecular lipid leaflet (see figure 3.7) corresponds to the lower limit indicated by the results of Rogers (1959),

Swift and Holmes (1965) and Robertson (1959, 1966).

Several factors combine to produce possible errors in the estimation of lipid content by solvent extraction - (1) The difficulty of obtaining a clean separation of protein and lipid from the proteolipid unit cell membrane is shown by the variation in published values for the protein:lipid ratio of human erythrocyte membranes; values of between 4:1 and 1:1 have been obtained [Stein (1967)]. (2) In the case of keratin fibres, some lipid may be bound by lipoprotein bonds which are resistant to solvent action. (3) The wool sample used in the present work had been extracted with petroleum ether, so a small amount of lipid may have been removed. (4) A small proportion of the extracted lipid may have come from nuclear remnants present in the cortical cells [Fraser et al. (1963)].

These possible sources of error tend to cancel each other, so a figure of 1% will be assumed for the lipid content of Merino wool.

Considerations of (1) the relative thicknesses of the stained and unstained layers of the cell membrane complex (figure 3.5), and (2) the weight of lipid extracted, suggests that the estimate of 3.5% for intercellular cement is of the right order of magnitude.

(d) Total content of cell membrane complex in Merino Wool

In accordance with the original definition by Birbeck

and Mercer (1957b), the 'cell membrane complex' is taken to include the two lipoprotein unit cell membranes from adjoining cells, plus the 'filling' of intercellular cement.

The following estimates have been made for the 'cell membrane' content of keratin fibres - 8% [Lundgren and Ward (1963); 1-2% [Bradbury and King (1967)]; 5-7% [Alexander and Earland (1950)]. Bradbury and King's estimate represented the lipid content plus a small amount of intercellular cement; Alexander and Earland's preparation contained resistant membrane material together with a considerable amount of cuticular and cortical contamination; Lundgren and Ward estimated their value by considering the relative thickness of Rogers' (1959) β- and δ-regions as seen in stained fibre cross-sections.

The results described in the preceding sections allow a much more direct estimation to be made. Thus, keeping in mind the definition of 'cell membrane complex' given above, the sum of the weights of resistant membranes (1.5%), lipid material (1%) and intercellular cement (3.5%) gives a total of approximately 6% for the weight of 'cell membrane complex' present in Merino wool.

3[D](v) A Note on Nomenclature of the Components of Cuticle Cells:

Lindberg et al. (1949) proposed the names epi-, exo- and endo-cuticle for the 3 layers of the cuticle cells of

animal fibres. They stated that "the nomenclature follows that which is customarily used in connection with the insect cuticle".

The cuticle of insects is a multi-purpose integument which forms a continuous extra-cellular layer over the entire body surface [see, e.g. Richards (1951); Locke (1964)]. Furthermore, the epicuticle of insects is not a single entity, as in keratin fibres, but consists of several layers with varying chemical and physical characteristics [see, e.g. Locke (1966)].

The results presented in this thesis confirm that epicuticle is neither extracellular nor continuous, so the name 'epicuticle' is not generically correct.

Lindberg et al. (1949) defined epicuticle as the membrane raised by chlorine water (presumably on the intact fibre, since they regarded it as a continuous external layer). What should the membrane raised on isolated cuticle cells be called? In the case of human hair, approximately five-sixths of the 'epicuticle' raised on isolated scales is not raised on the intact fibre, because this fraction of the membrane occurs under the next several overlapping scales. Also, does epicuticle cease to be epicuticle when it passes from the top side to the underside of the cuticle cell?

The names 'exocuticle' and 'endocuticle' also give

the (incorrect) impression of continuity over the entire fibre surface. This point was originally made by Alexander et al. (1963, page 7).

The original nomenclature of Mercer and Rees (1946) could perhaps warrant reconsideration i.e. K_1 and K_2 phases were used to describe, respectively, exocuticle and endocuticle. The term 'cuticle cell membrane' or simply 'cell membrane' would overcome the misconceptions inherent in the name 'epicuticle'.

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4. SOME ASPECTS OF DIFFUSION IN KERATIN FIBRES.

[A] INTRODUCTION:

The preceding chapters have emphasised that keratin fibres consist of small packets of protein material, each wrapped in a keratinised unit cell membrane, the whole being held together by an intercellular cement. In living systems, the cell membrane functions as a barrier preventing the intracellular fluid from mixing rapidly with the external environment [see, e.g., Schoffeniels (1967)]. The physical structure of the unit cell membrane still exists in the fully-keratinised fibre [Birbeck and Mercer (1957a,b); Fraser et al. (1963); Rogers (1964a)]. Molecules passing into or out of each individual cell must therefore pass through the unit cell membrane unless this has been removed or modified by chemical pretreatment. It is reasonable, then, to expect that the cell surfaces, and the cell membrane complex, may influence the movement of molecules through the fibre as a whole.

The Allworden and Herbig reactions demonstrate the

semi-permeable properties of the external cuticle cell membranes, while the present work equates this 'epicuticle' with whole-fibre cell membranes. Hence the possibility remains that the cell membranes form a barrier to diffusion of certain types of molecules; however because of the unequivocal demonstration of the non-continuous nature of 'epicuticle' (chapters 2 and 3), it is no longer essential to consider this external membrane as a <u>whole-fibre</u> barrier to diffusion.

Any reaction between a liquid and a solid involves a number of steps, the slowest of which determines the overall rate of reaction. Although the fine diameter of wool fibres results in an extremely high specific surface area, many reactions are apparently controlled by diffusion through the external surface [Alexander et al. (1963), page 128]. Since 'epicuticle' is now known to be discontinuous, perhaps considerations of the total surface area of the individual cells rather than of the external surface of the fibre, may affect the interpretations of some diffusion phenomena. The surface area of a 20μ-diameter fibre (such as Merino 64's wool) is approximately 2000 sq. cm/cc, while the total surface area of the individual cells, calculated from the data in chapter 3, is 25,000 - 50,000 sq. cm/cc. The relative rates of transport <u>to</u> the cell surfaces and of diffusion <u>through</u> the surfaces will still determine the

overall reaction rate, but the chances that diffusion through the cell membrane will be the limiting factor, are considerably lessened.

In the present chapter, the above considerations are discussed in terms of results for extraction of proteins from Merino wool and for diffusion of alcohols into modified wool. A brief discussion and re-evaluation of some data taken from the literature, in relation to the concept of non-continuous 'epicuticle', are also included.

[B] MATERIALS AND METHODS:

(i) Wool:

Merino 64's wool was from the same sample as that used for the work described in chapters 2 and 3.

(ii) Extraction of Protein Material with Formic Acid:

Wool was gently agitated in 98-100% formic acid for 2 x 30 minute periods at 20°C (50:1 liquor:wool ratio). The combined formic acid solutions were filtered, freeze-dried, and extracted with 2:1 (v/v) chloroform-methanol to remove lipid material. This gave a protein probably contaminated with some inorganic material [Bradbury et al. (1965a)].

(iii) Isolation of Proteins Dissolved by Chlorine Water:

The residual wool from the formic acid extraction was immersed in saturated chlorine water at 30:1 liquor:wool

ratio and 20°C for 2 minutes, then quickly filtered.

(a) The filtrate was immediately frozen in dry ice-alcohol, freeze dried to remove chlorine water and the residue dissolved in formic acid. After centrifuging to remove insoluble material, the formic acid solution was freeze-dried, resulting in a preparation of protein material assumed to have passed through the Allworden membrane.

(b) The residual wool was immediately transferred to a large volume of 0.5% sodium bisulphite solution (antichlor), washed 4 times in distilled water, then shaken vigorously on a laboratory shaker. The mechanical agitation ruptured the Allworden sacs, releasing the 'osmotically active' protein solution. The resulting aqueous solution/suspension was evaporated to dryness under vacuum, dissolved in formic acid, and the insoluble material removed by centrifugation. The membrane-impermeable protein material was recovered by freeze-drying.

Care was taken that the proteins prepared under (a) and (b) had spent as closely as possible to the same time in contact with both chlorine water and formic acid, in order to equalise the amount of chemical degradation occurring after initial separation into membrane-permeable and membrane-impermeable proteins.

(iv) Estimations of Molecular Weights of Proteins:

The isolated proteins were subjected to molecular weight estimations by Sephadex gel-filtration, using essentially the method described by Andrews (1964).

Approximately 30 mgm of protein was dissolved in 8M urea buffered to pH 6.8 with 0.01 M tris (hydroxymethyl) amino methane/hydrochloric acid, and eluted through a 60 cm x 2.6 cm Sephadex G-200 column* using the same solvent. The eluent was separated into fractions on a fraction-collector, and the proportion of protein in each fraction was estimated photometrically at 280 mμ. The column had previously been calibrated with proteins of known molecular weight.

(v) Measurement of n-Propanol Absorption:

The rate of absorption of n-propanol vapour, under anhydrous conditions, was measured on several of the modified wools listed in section 2[D]. These measurements were made by Dr. I. C. Watt, at C.S.I.R.O. Division of Textile Physics, Ryde, N.S.W. The apparatus consisted of a quartz spiral spring balance mounted in a vacuum system, the whole being enclosed in an efficient air-thermostat at 35°C [Watt (1960, 1964)]. Absorption was allowed to proceed, on the initially-dry sample, by intermittent admission of n-propanol vapour to keep the system at close to saturation vapour pressure.

* [The author is indebted to Mr. J. M. O'Shea for making available the calibrated Sephadex G-200 column and ancillary equipment].

[C] RESULTS AND DISCUSSION:

4[C](i) Diffusion of Protein Molecules out of Merino Fibres:

Results of the gel-filtration experiments described above are summarised in figure 4.1.

The protein extracted by formic acid is assumed to come from the intercellular cement (see chapter 3). Bradbury et al. (1965a) came to the reasonable conclusion that this material would be of low molecular weight. The large proportion of high molecular weight material (500,000 and above) is therefore rather surprising. The high molecular weight proteins probably represent intercellular cement, while the material of lowest molecular weight may have diffused through the cell membranes as a result of the swelling and disaggregating action of formic acid on the cell contents. The formic acid probably also alters the permeability of the unit cell membranes. It is unlikely that proteins of molecular weight around 500,000 would diffuse through the membranes. Amino acid analyses on this high molecular weight fraction should prove interesting, especially since this fraction must have a low cystine content (see the analytical data for the total formic acid extract, table 3.7, page 157). Apparently, then, the high molecular weight is not due to the presence of a large number of disulphide cross-links.

The estimation of amount of intercellular cement

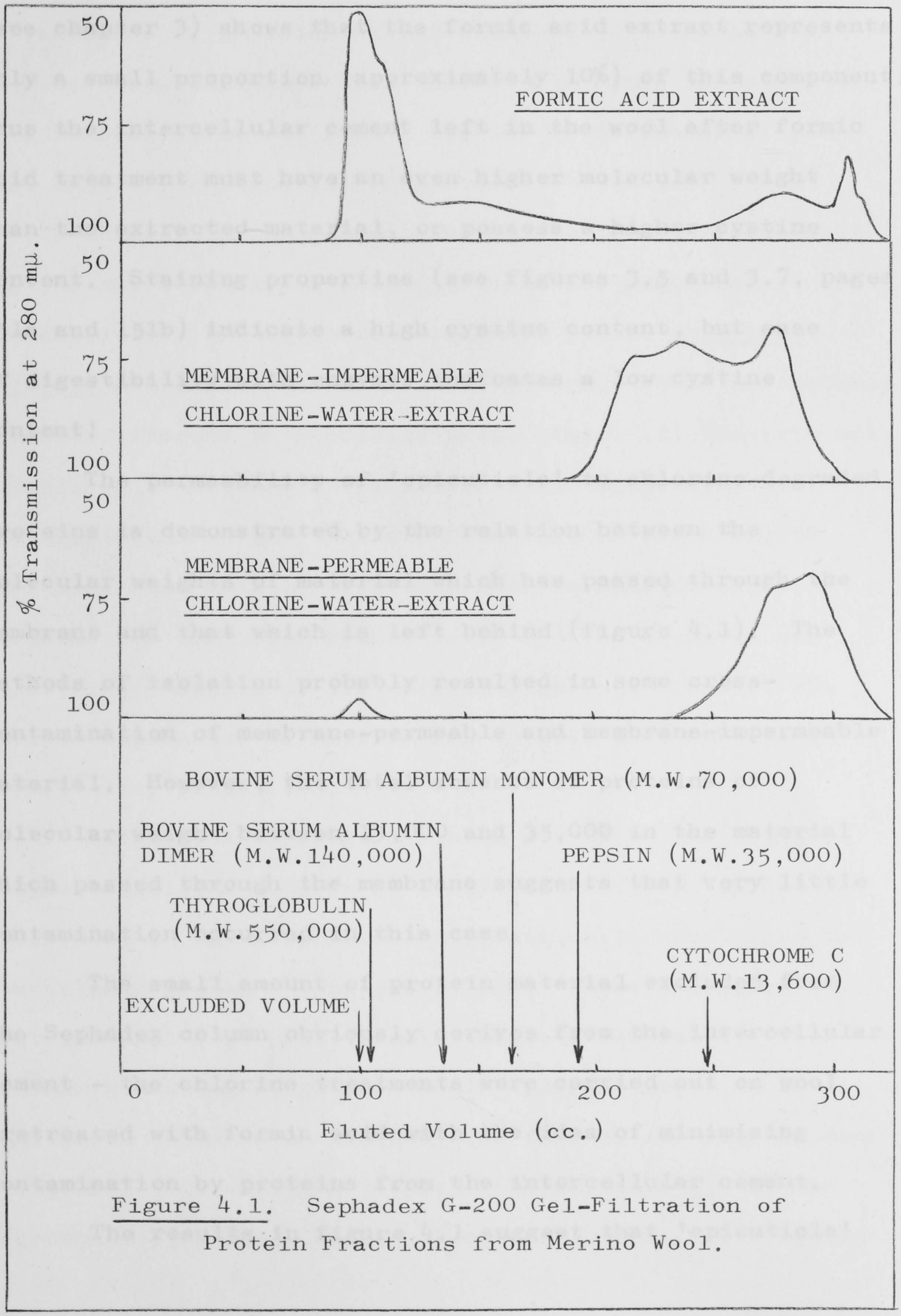

Figure 4.1. Sephadex G-200 Gel-Filtration of Protein Fractions from Merino Wool.

(see chapter 3) shows that the formic acid extract represents only a small proportion (approximately 10%) of this component. Thus the intercellular cement left in the wool after formic acid treatment must have an even higher molecular weight than the extracted material, or possess a higher cystine content. Staining properties (see figures 3.5 and 3.7, pages 151a and 151b) indicate a high cystine content, but ease of digestibility with enzymes indicates a low cystine content!

The permeability of 'epicuticle' to chlorine-degraded proteins is demonstrated by the relation between the molecular weights of material which has passed through the membrane and that which is left behind (figure 4.1). The methods of isolation probably resulted in some cross-contamination of membrane-permeable and membrane-impermeable material. However, the total absence of proteins of molecular weight between 13,000 and 35,000 in the material which passed through the membrane suggests that very little contamination occurred in this case.

The small amount of protein material excluded from the Sephadex column obviously derives from the intercellular cement - the chlorine treatments were carried out on wool pretreated with formic acid with the idea of minimising contamination by proteins from the intercellular cement.

The results in figure 4.1 suggest that 'epicuticle'

is permeable to proteins of molecular weight as high as 13,000. This indicates that 'epicuticle' (and the other cell membranes) do not form a barrier to diffusion of the vast majority of chemical reagents.

However, 3 things must be kept in mind before accepting the above result as definite value for membrane-permeability. (1) These results refer to the chlorine-modified membrane - the unmodified membrane would undoubtably exhibit different permeability properties. (2) The external cuticle cell membrane ('epicuticle') has apparently lost its bimolecular lipid leaflet. The results which follow will show that this material can exert a considerable influence on diffusion properties. (3) The chemical and 'electrical' nature of the diffusing molecules and of the various components of keratin fibres will also influence permeability properties.

Extraction of keratin fibres with alkaline thioglycollate solutions removes 60 - 85% of the fibre, as the first step in the separation and fractionation of high- and low-sulphur proteins (see table 3.2). These extracted proteins must also pass through the cell membranes. The minimum molecular weight of the low-sulphur proteins is considered to be around 10,000, and that of the high-sulphur proteins 22,000 - 27,000 [Crewther et al. (1965)], so the permeability of the alkaline thioglycollate-modified membranes

is not inconsistent with that found for chlorine-modified membranes. Similar permeabilities would apply for alkaline extraction of low- and high-sulphur fractions from oxidised wool (i.e. for the α-keratose and ϒ-keratose fractions, respectively).

The above approach could be usefully extended to include studies of the proteins extracted, e.g. by chemical shrinkproofing reagents, but some other technique is obviously necessary to measure the permeability of the unmodified membranes.

4[C](ii) Diffusion of n-Propanol into Merino Fibres:

Because of the discontinuous nature of 'epicuticle', diffusion of substances into keratin fibres can now be considered to occur by two different mechanisms. (1) Through the 'epicuticle', then through the 'a' and 'b' layers of the exocuticle, the endocuticle, the inner cell membrane, the intercellular cement, and finally into the main body of the fibre. (2) Along the cell membrane complex between the overlapping cuticle cells.

It seems reasonable to assume that many types of sorbate molecules would rather traverse the cell membrane complex than diffuse through the many layers of differing chemical and physical structure which occur in cuticle cells. This would apply particularly to diffusion into fibres such

as human hair, where a molecule entering the fibre via method (1) would have to pass through as many as 6 cuticle cells before entering the cortex.

The rate at which molecules penetrate dry wool decreases with increasing molecular size of the sorbate [Bradbury and Leeder (1963)]; while for a particular sorbate, the rate of uptake can be varied by application of chemical pretreatments [Leeder and Lipson (1963)]. The difference in uptake rate between modified wool and normal wool is accentuated by increasing the molecular size of the sorbate [Leeder and Watt (1969)].

In the present work, the rate of uptake of n-propanol was measured on several of the modified wools described in section 2[D]. The results obtained are presented in figure 4.2, where weight % uptake of n-propanol vapour is plotted against $(\text{time})^{1/2}$.

Surprisingly, 'surface' modifications, such as permanganate/salt shrinkproofing and sulphuryl chloride treatment, produced only small changes in uptake rate. Lipid material is extracted from the wool by treatment with formic acid [see chapter 3] and ethanol [Anderson and Leeder (1965b)]. The large increases in rate of sorption of n-propanol indicates that the bimolecular lipid leaflets of the unit cell membranes control the rate of uptake of these molecules to a much greater extent than does the fibre surface!

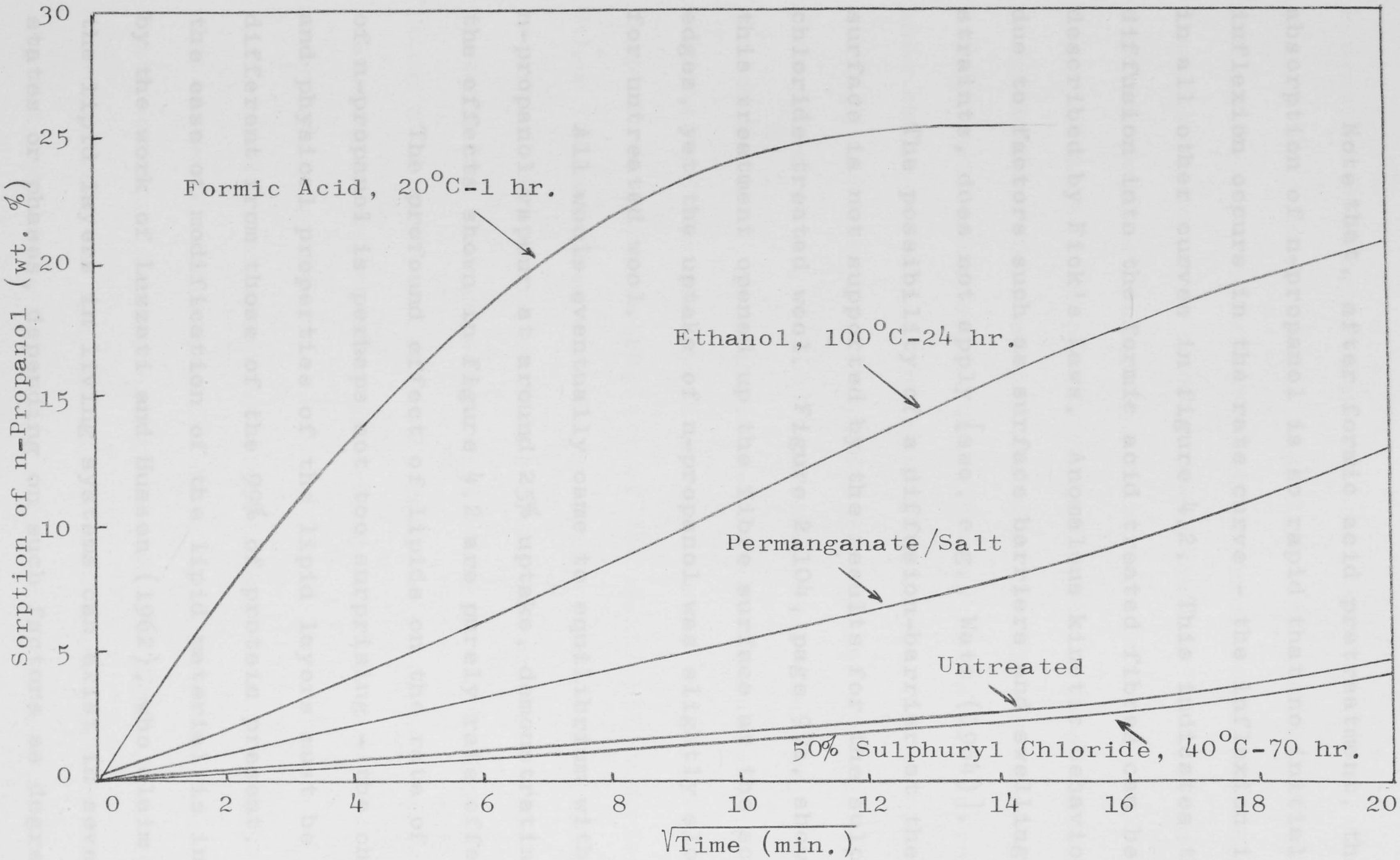

Figure 4.2. Rate of Uptake of n-Propanol Vapour by Modified Merino Wools.

Note that, after formic acid pretreatment, the absorption of n-propanol is so rapid that no initial inflexion occurs in the rate curve - the inflexion is present in all other curves in figure 4.2. This indicates that diffusion into the formic acid treated fibres can be described by Fick's Laws. Anomalous kinetic behaviour, due to factors such as surface barriers and swelling constraints, does not apply [see, e.g., Watt (1964)].

The possibility of a diffusion-barrier at the fibre surface is not supported by the results for the sulphuryl chloride treated wool. Figure 2.104, page 97a. showed that this treatment opened up the fibre surface at the scale edges, yet the uptake of n-propanol was slightly slower than for untreated wool.

All wools eventually came to equilibrium with the n-propanol vapour at around 25% uptake, demonstrating that the effects shown in figure 4.2 are purely rate effects.

The profound effect of lipids on the rate of sorption of n-propanol is perhaps not too surprising - the chemical and physical properties of the lipid layers must be very different from those of the 99% of protein present. Further, the ease of modification of the lipid material is indicated by the work of Luzzati and Husson (1962), who claim that the lipid layers in living systems can exist in several states or phases, depending on such factors as degree of

hydration and temperature.

The above results highlight the need to study the role of lipids as a barrier to diffusion of other molecules. Thus it is hoped to extend these studies to include aqueous systems such as dyeing.

Realisation of the importance of lipids in alcohol sorption may offer an explanation for the incompletely-understood mechanism by which dyeing rates are increased when small amounts of organic solvents, such as benzyl alcohol and butanol, are added to aqueous dye solutions. [see Giles et al. (1962) and references therein]. Interaction between the organic solvent and the lipid layers could alter the transport properties of the cell membrane complex or the permeability of the unit cell membranes. Other factors must also be operative, however, since Giles et al. (1962) found evidence of a 'solvent-assisted ' dyeing effect on gelatin, while Medley and Ramsden (1960) found that addition of small amounts of organic solvent resulted in an increase in dyeing rate of keratin films made from cow horn.

Medley and Andrews (1960) suggested that solvent pretreatments increased the rate of dye uptake by removing fatty material from the interior of the fibre, and that solvent-assisted dyeing had the additional effect of increasing the mobility of the dye within the fibre. It may not be

necessary to separate these two effects, however, since an increase in permeability of the internal cell membranes, by modification of the lipid layers, would manifest itself as an apparent increase in mobility of the dye within the fibre.

The rapid low-temperature dyeing of wool in concentrated formic acid [Harrap (1959), Maclaren (1960)] probably also depends, at least in part, on disruption and extraction of lipid material.

4[C](iii) Sorption from Aqueous Media - Re-interpretation of Published Data:

Undamaged wool fibres show non-Fickian absorption in the early stages of dyeing from aqueous solution i.e. there is an initial (negative) deviation from linearity in the plot of dye uptake versus $(\text{time})^{1/2}$ [Lindberg (1950); Peters and Lister (1954); Medley and Andrews (1959)].

These results, together with observations that dyeing rates increased after chemical or mechanical damage to the fibre (see later) indicated that some type of surface barrier to diffusion was operative. Since 'epicuticle' was considered to be a continuous outer membrane, it was universally identified with this surface barrier. According to Gralen (1950), "when the epicuticle is damaged, the dye penetrates through the holes and stains the keratin of the fibre".

In the introduction to this chapter, the suggestion

was made that penetration of sorbates could occur by means of the cell membrane complex, rather than (or as well as) through the cuticle cells. This does not, of course, exclude the possibility that the cell membranes around the individual cuticle and cortical cells form a barrier to passage of certain molecules into or out of the cells. A few examples from the literature will be discussed in terms of the above concepts.

Whewell and Woods (1944) found that mechanical damage to wool fibres increased the rate of uptake of Methylene Blue. These authors noted that the appearance of the dyed fibres gave the impression of damage to a superficial skin covering the scales.

The photomicrographs published by Whewell and Woods show that staining always starts at the scale edges, the coloured area gradually increasing until the whole fibre is stained. It is now suggested that penetration of dye occurred between the scales, which had been disrupted by the mechanical treatment. A positive Allworden reaction on fibres treated in a similar manner to those of Whewell and Woods, was shown in figure 2.112, page 104a - this photomicrograph also shows considerable disruption of the scales, supporting the present interpretation.

Lindberg et al. (1949) interpreted the observation of a progressive increase in rate of dye uptake as wool

passed through the various manufacturing processes [Royer et al. (1947)] as a "progressive impairment in the barrier offered by the epicuticle." But again the effect can be explained in terms of partial disruption of the whole cuticle structure.

The greatly increased penetration of dyes through both the outer and inner surfaces of a knotted fibre [Millson and Turl (1950)] is a particularly good demonstration of the effect of scale disturbance on dyeing properties - bending a fibre in this way results in partial separation of the individual scales, but would not be expected to rupture the membranes surrounding each cuticle cell.

The work of O'Reilly et al. (1952) supports the above conclusions. Observations of dyeing and staining of processed fibres led these authors to suggest that "the epicuticle may actually surround each scale individually and leave some uncovered areas beneath the scales through which dyes could easily penetrate when the scale is lifted." The conclusions of O'Reilly et al. were subsequently discredited by Lagermalm et al. (1953) on the basis that epicuticle was a continuous external membrane.

The increased dyeing rate of chemically-modified fibres also appears initially as a staining of the scale edges [see, e.g., Millson and Turl (1950)]. The lability of the intercellular cement (table 3.8, page 158) and the

ease of extraction of lipid material with organic solvents, suggests that chemical attack would occur more readily in these regions than within the cells or the resistant membranes.

Lindberg (1953a,b,c,d) studied the effect of alcoholic potassium hydroxide on many physicochemical properties of wool, and concluded that changes in permeability and/or polarity of epicuticle were responsible for the observed changes in surface properties. However, Lindberg's results [see figures 1-6 (1953c)] show that, although alcoholic alkali treatment greatly increased the rate of dye uptake, an essentially non-degradative alcohol extraction also greatly increased the rate of uptake of the several dyes studied. This is again consistent with the view that the lipid layers contribute significantly to the sorption behaviour of keratin fibres.

In the light of the preceding discussion, the cuticular barrier to diffusion suggested by Kopke and Nilssen (1960), is probably influenced by the degree of disturbance of the scales and by the state of the cell membrane complex. Another factor to consider is that the cuticle of wool fibres is significantly less polar than the cortex [Bradbury et al. (1965b, 1966)], so may tend to absorb smaller amounts of polar and charged molecules than the cortex.

As early as 1936, Speakman and Smith suggested that the cuticle was a barrier to acid dyestuffs, and that dye absorption may take place mainly through the intercellular phase. Since the late 1940's, preoccupation with the role of (continuous) epicuticle as a surface barrier to diffusion apparently prevented acceptance of this idea. It now seems that Speakman and Smith's original interpretation was correct.

An increased rate of sorption of hydrochloric acid by wool modified with alcoholic alkali, was attributed to a change in the permeability of 'epicuticle' [Lindberg (1950, 1953c)]. Whether the outer membranes are continuous or not, surely they would not be expected to retard the penetration of H_3O^+ and Cl^- ions, no matter how heavily hydrated these ions were! Changes in polarity and acid-base characteristics of the entire cuticle are more likely to be the controlling factors. Thus Wikstrom et al. (1955) suggested that similar types of surface modifications change the polarity of the wool to a depth considerably greater than the 'mathematical' surface.

Several other literature reports can be interpreted to support the concept that the cell membrane complex is involved in diffusion phenomena. Figure 2 of the paper by Bradbury et al. (1963) is a cross-section of a Lincoln fibre pretreated with potassium permanganate - the permanganate has diffused into the fibre preferentially at the scale

junctions. Swift and Holmes (1965) found increased penetration and deposition of phosphotungstic acid in the cell membrane complex of human hair cuticle following ethanol-extraction. Ethanol extraction also increases the rate at which papain-bisulphite disrupts human hair [Holmes (1964a,b)].

The preceding results and discussions show that the cell membrane complex participates in many diffusion processes. Two separate effects may be involved - (1) molecules may diffuse <u>along</u> one or more of the various components of this complex, and (2) the lipid- and protein-layers of the unit cell membranes may be acting as surface barriers to diffusion <u>into</u> the individual cells.

The relative importance and degree of interaction of these two effects will vary with extent and type of pretreatment and with the chemical nature of the diffusing molecule. Not enough is known about the cell membrane complex to allow more definite conclusions; the present results suggest that a closer study of the chemistry and physics of this complex, especially in relation to diffusion phenomena, will be very rewarding.

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5. APPENDIX.

SEPARATION AND ANALYSIS OF EXO- AND ENDO-CUTICLE.

[A] INTRODUCTION:

The work described in the previous chapters has helped to characterise the physical and chemical structure of the cuticle cell membranes, but little is known about the physical structure or the chemistry of the other layers which make up the cuticle cell. Bradbury et al. (1965b) have determined the amino acid composition of whole cuticle, but the only component which has been isolated and analysed is epicuticle [King and Bradbury (1967)]. To further our understanding of transport processes through the surface layers of keratin fibres and to help interpretation of other surface phenomena, a greater knowledge of the properties of exo- and endocuticle would seem to be desirable.

The only information available on these cuticle components is that obtained from staining and digestion studies, using the electron microscope as an analytical

tool. Mercer (1955) reported that, in untreated fibres, endocuticle is more dense to electrons than exocuticle, but that after reducing and staining for sulphydryl groups with osmium tetroxide, the exocuticle is more dense, particularly the region now known as the 'a' layer.

Electron microscopy has also shown that tryptic digestion preferentially removes endocuticle, while oxidation with peracetic acid followed by dilute ammonia extraction leaves the (non-keratinous) endocuticle and cell membrane material. Thus Mercer concluded that the exocuticle consists of keratinous protein stabilised by disulphide cross-links, and the endocuticle of non-keratinous protein. The ease of digestion of this component by proteolytic enzymes is a direct consequence of the low cystine content. Since the electron microscope shows that the endocuticle of untreated fibres is denser than keratin, Mercer also suggested that this fraction may contain other constituents, such as phosphates from cell debris.

A large number of electron-microscopic investigations by various workers produced results in agreement with those of Mercer, and it is generally accepted that exo- and endocuticle are present in approximately equal amounts, i.e. they each constitute about 5% of the wool fibre.

Very little else is known about exo- and endocuticle. Lagermalm (1954) appears to have isolated exocuticle +

epicuticle by heating wool in phenol at 100°C for 2 hours followed by tryptic digestion; Swift and Holmes (1965) showed by electron microscopy that the endocuticle of human hair was removed by treatment with papain/bisulphite; and treatment with hydrochloric acid at pH 2 and 100°C was reported to preferentially extract endocuticle [Leach et al. (1964)]. Mercer and Rees (1946) found that the resistance of endocuticle to enzymatic digestion could be increased by cross-linking with formaldehyde - another indication of the lack of cross-links in this component.

Fraser and Rogers (1955c) postulated that ridges observed on isolated scale fragments result from a two-component 'fibril + cementing substance' structure of endocuticle, but this has yet to be confirmed experimentally.

Considering the amount of work done on enzymatic digestion and electron microscopy of the wool fibre cuticle, it is perhaps surprising that exo- and endocuticle have not yet been separated and analysed. In the present work, the whole cuticle was isolated, then further separation into exo- and endocuticle fractions was attempted using various enzymes.

[B] MATERIALS AND METHODS:

(i) Preparation of Cuticle Material [Bradbury et al. (1965b)]:

1.5 gm samples of Merino 64's wool were cut into approximately 0.5 cm lengths and agitated for 1 hour in 50 ml

re-distilled 98-100% formic acid, using a Vibromix agitator at maximum amplitude. Prior agitation for 2 x 5 minute periods in formic acid was necessary to dislodge and remove contaminating skin flakes from the fibre surface. The suspension of cuticle material was filtered through a 35-mesh stainless steel sieve to remove intact wool fibres. The cuticle suspension was centrifuged to remove the formic acid, and washed six times at the centrifuge with ethanol. Cortical cells and other contaminants were removed by filtering through a 50-mesh nylon sieve followed by an 18μ stainless steel sieve. Any very small contaminants denser than wool were then removed by centrifuging through carbon tetrachloride, and lighter contaminants by layering the ethanolic suspension on ethylene glycol. After finally washing in ethanol, the residual cuticle material was centrifuged, dried in the air overnight, then vacuum-dried and weighed.

Thirteen such operations yielded 183 mgm of pure cuticle, which represented 1% of the weight of wool originally used.

(ii) Enzymatic Digestions:

(a) Trypsin: Approximately 20 mgm samples of cuticle were dried and accurately weighed. 2.0 ml of 0.2% trypsin solution was added and the mixture kept at 40°C with occasional shaking for the required time. The trypsin

solution was made by dissolving pure trypsin (Worthington 2 x recrystallised) in pH 8.4 borate buffer. These conditions are essentially those used in the original cuticle-digestion studies of Mercer and Rees (1946).

(b) Papain: The same conditions as those for trypsin were used, the papain again being Worthington 2 x recrystallised grade.

(c) Pepsin: Similar conditions to those for trypsin and papain were used for pepsin treatments, except that the 2 x recrystallised pepsin was dissolved in 0.1 M sodium acetate solution adjusted to pH 1.2 with hydrochloric acid.

Extreme difficulty was experienced in wetting-out the cuticle powder with the enzyme-buffer systems, and even with water. This was eventually overcome by wetting the cuticle sample in formic acid, and replacing the acid with water and then enzyme solution at the centrifuge.

After enzyme treatment for the desired time, residual cuticle material was recovered by centrifuging, washed with water, dried in vacuo at 20°C and weighed. Amino acid analyses were carried out on whole cuticle, cuticle residue, and the resultant mixture of enzyme + digested cuticle material.

(d) Pronase digestions on whole-wool [Springell (1963)]:

2 gm samples of wool were treated with 20 mgm of pronase (K.and K laboratories) in 400 ml pH 8 ammonium

acetate/ammonia buffer (containing 10% (v/v) ethanol as bactericide) at 37°C for periods of between 1 and 6 weeks.

(iii) Amino Acid Analyses:

Amino acid analyses of the various cuticle fractions were carried out as described in section 3[C](iv).

[C] RESULTS AND DISCUSSION:

Since three different enzymes at two different pH conditions were used, wide variation was expected in amount of material digested. However, the preliminary results in table 5.1 show that approximately the same weight of protein was removed in each case.

TABLE 5.1.

DIGESTION OF WOOL CUTICLE WITH ENZYMES AT 40°C.

Enzyme	Time of digestion (days)	Weight loss (%)
Trypsin, pH 8.4	1	23
" " "	4	22
" " "	7	20
Papain, pH 8.4	7	20
Pepsin, pH 1.2	10	26

It is also significant that, in the case of trypsin, dissolution of protein ceased after less than 1 day. This indicates that a specific fraction of the wool cuticle is

susceptible to trypsin; if further results confirm that other enzymes have the same effect, this may mean that a definite histological component is extracted.

The amino acid composition of three cuticle fractions are compared in table 5.2, for the 7 day trypsin treatment in which 20% of material was extracted.

TABLE 5.2.

AMINO ACID ANALYSES OF MERINO CUTICLE PREPARATIONS (MOLE %)

Amino Acid	Whole Cuticle	Tryptic Residue	Tryptic Digest
Alanine	5.66	5.98	6.36
Arginine	4.97	3.81	4.42
Aspartic acid	3.58	3.24	15.13
Citrulline	0.89	1.25	0.28
Cystine/2.[a]	15.75	16.05	0.0
Glutamic acid	9.35	9.36	12.63
Glycine	10.91	10.31	15.68
Histidine	1.11	0.99	0.87
Isoleucine	2.34	2.41	4.48
Leucine	5.92	5.96	8.32
Lysine	2.39	2.50	4.94
Methionine	0.41	0.33	1.52
Phenylalanine	1.92	1.97	2.07
Proline	4.75	4.40	3.92
Serine	14.82	17.05	7.78
Threonine	5.05	4.98	4.65
Tyrosine	3.04	3.27	4.03
Valine	6.55	6.11	3.02
Anhydroamino Acid Recovery	93.7%	95.6%	58.9%

[a] Includes oxidised form (cysteic acid).

The low yield obtained for the trypsin digest resulted from loss of an unknown amount of material during preparation for analysis - the relative proportions of amino acids and the validity of the results are not affected. Published amino acid analyses for trypsin [Cunningham (1965)] were used to correct for the known proportion of enzyme in the preparation. Until many more similar experiments are carried out it is probably advisable to resist the temptation to critically analyse and compare the results. However, several features are worthy of comment.

In particular, the content of aspartic acid + glutamic acid is increased two-fold in the extracted material, and the proportion of all polar groups is increased 25%. The residual cuticle is therefore less polar than whole cuticle, which Bradbury et al. (1965b) have shown to be already less polar than whole wool.

The most striking feature of the results in table 5.2 is the complete absence of cystine in the protein material extracted by trypsin. Endocuticle is defined as the region which does not stain with metals after reduction of cystine to cysteine showing that very little cystine is present in this region. This indicates that at least part of the endocuticle has been extracted with trypsin - electron microscopy on cuticle and further gravimetric analyses after various enzymatic digestions should show whether the

endocuticle is only 20% of the cuticle, as the present results suggest, or whether the two-component structure for endocuticle proposed by Fraser and Rogers (1955c) is perhaps applicable.

There is considerable confusion in the literature regarding dissolution of wool by enzymes [see, e.g. Alexander et al. (1963)] - only approximately 10% of untreated wool is digested by most proteolytic enzymes but considerable dissolution and disruption occurs after prior chemical degradation. In particular, reagents which attack cystine result in rapid enzymatic digestion of the treated wool, obviously because the enzyme can then attack keratinous protein material.

Unfortunately, the different behaviour of untreated and (cystine-) modified wools is not generally recognised. Geiger (1944a, 1944b) is often quoted as having analysed scale material isolated by pepsin digestion of reduced and alkylated wool fibres, while Holmes (1964a) isolated scale cells from human hair as "very thin plates which curl up" by papain/bisulphite digestion. Qualitative papain/bisulphite digestions were carried out during the present work - the wool first broke down to give cortical and cuticle cells; the cuticle cells became thinner with time until, after about 1 week, very thin fragments (epicuticle?)

remained and even these eventually disappeared. Thus the cuticle material isolated by Holmes does not have the 'substantial' appearance of the cuticle cells isolated from human hair during the present work (see figures 2.43 and 2.44, page 62b).

The use of reagents which break disulphide bonds, in conjunction with enzymes, does not appear to be a satisfactory method for isolation of wool components. Conversely, the present results indicate that non-keratinous material - defined here as protein containing no disulphide cross-links - can be selectively extracted from wool by pure enzymes.

Figure 5.1 shows the results for digestion of untreated wool with pronase. The graph levels off at around 12% dissolution. The weight of extracted material is approximately the amount expected for the total of endocuticle, intercellular cement and nuclear remnants [2 - 2.5% for endocuticle (see above); 3.5% for intercellular cement (chapter 3); and 6% for nuclear remnants and cell debris (see table 1.2)]. Similar experiments using other proteolytic enzymes, coupled with amino acid analyses, are needed to show whether enzyme-digestions provide a method for 'clean' separation of fibres into keratin and non-keratin fractions. Some differences in weight of protein extracted and in analytical figures would be expected

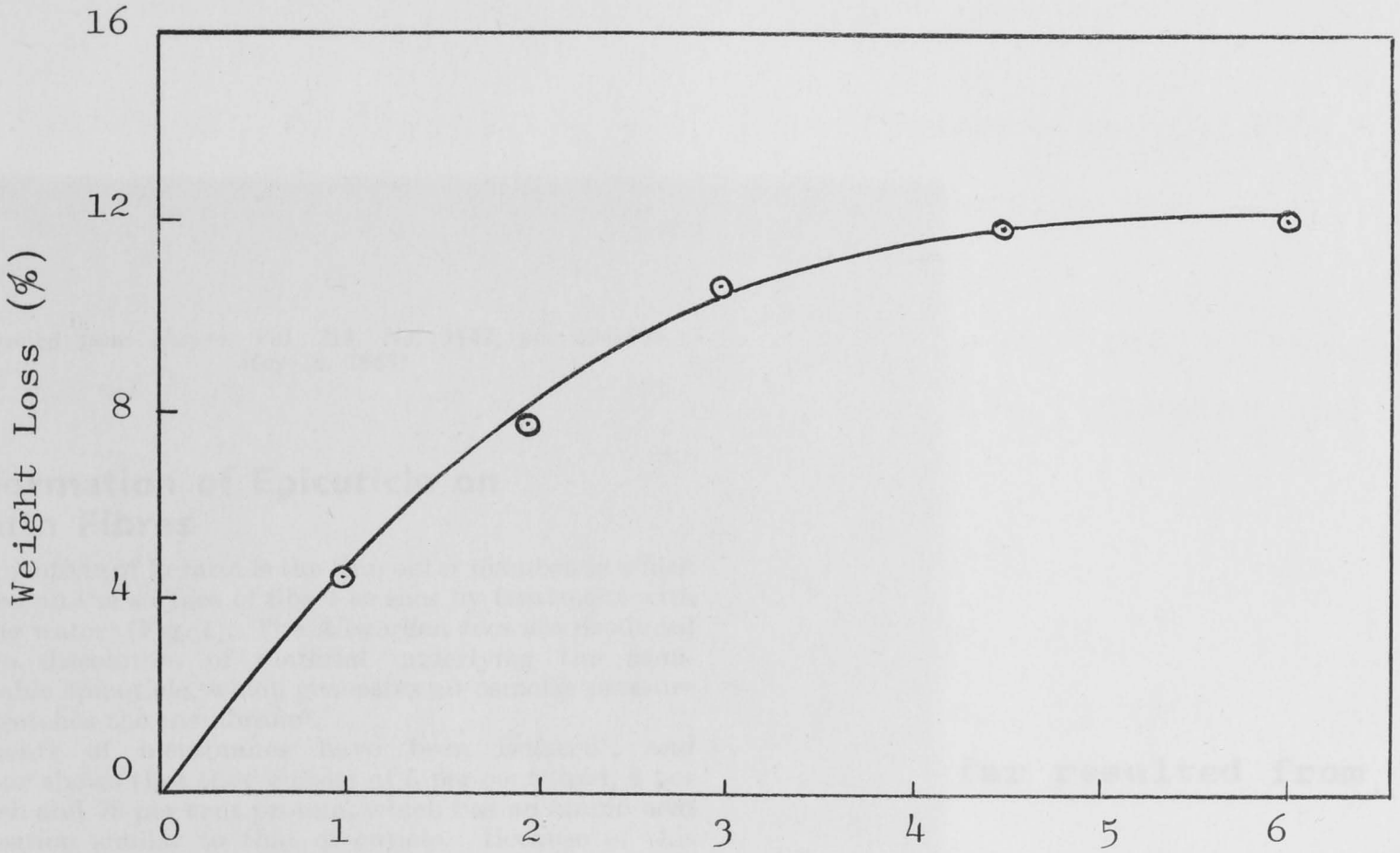

Figure 5.1. Digestion of Untreated Merino Wool with Pronase at 37°C.

because of the different peptide-bond specificities of the various enzymes.

Molecular weight determinations on the digested proteins, and comparisons of rate and/or extent of extraction with molecular weight of the enzyme should also give information on the permeability of the cell membranes.

(*Reprinted from Nature, Vol.* 218, *No.* 5142, *pp.* 694–695, *May* 18, 1968)

Conformation of Epicuticle on Keratin Fibres

THE epicuticle of keratin is the thin outer membrane which is raised on the surface of fibres as sacs by treatment with chlorine water[1] (Fig. 1). The Allwörden sacs are produced by the dissolution of material underlying the semi-permeable epicuticle, which generates an osmotic pressure and stretches the membrane[2].

Fragments of membranes have been isolated[3], and analysis[4] shows that they consist of 5 per cent lipid, 4 per cent ash and 78 per cent protein, which has an amino-acid composition similar to that of cuticle. Because of this unexceptional composition, it is surprising to find, in agreement with others[5,11], that the epicuticle is still intact in Merino wool even after severe treatments with alkali, acid, oxidizing and reducing agents and enzymes.

Our results concern the controversy over whether epicuticle is a continuous external membrane covering the whole of the fibre[3,6–9] but possibly folded under the scale edges[8], or whether it is discontinuous in the sense that each cuticle cell is covered by a separate membrane[10–12]. These studies have been made on a wide range of keratin fibres.

Treatment of wool with formic acid at room temperature causes dissolution of material from the cell membrane complex, and cuticle fragments are liberated by concurrent shaking or ultrasonication[13–14]. Treatment at 100° C for 1 h followed by gentle agitation detaches partially or completely whole cuticle cells from keratin fibres. A Merino fibre treated in this way and subsequently immersed in chlorine water is shown in Fig. 2. There are Allwörden sacs on partially detached cuticle cells. In

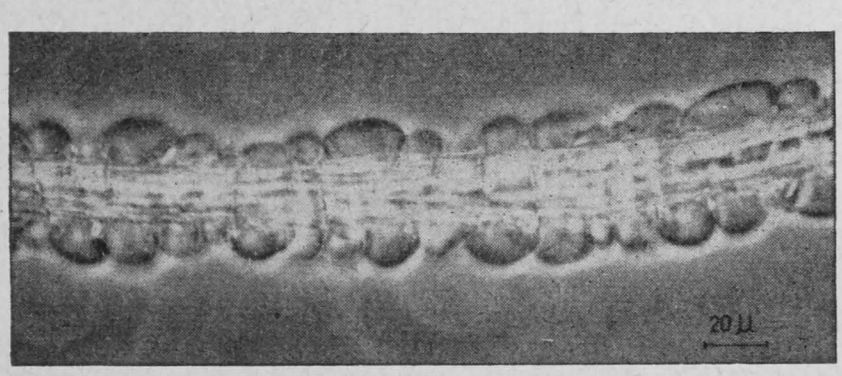

Fig. 1. Photomicrograph under phase contrast of a Merino fibre treated with saturated chlorine water.

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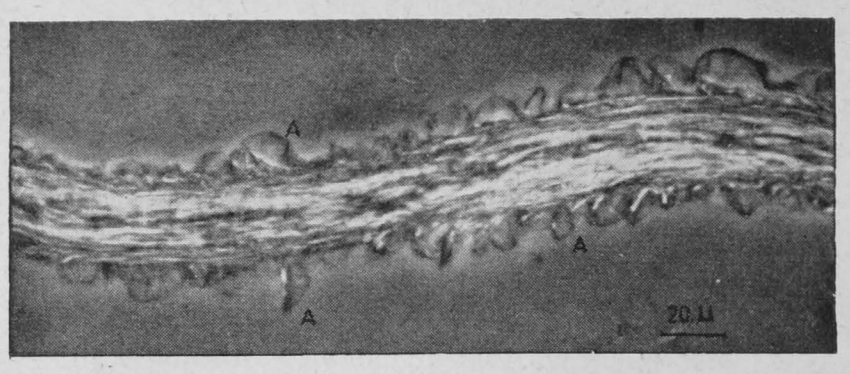

Fig. 2. Phase contrast photomicrograph of a Merino fibre heated at 100° C in formic acid for 1 h and gently agitated to loosen the cuticle. The fibre was then washed with water and treated with saturated chlorine water to produce Allwörden sacs, shown on partially detached cuticle cells at A.

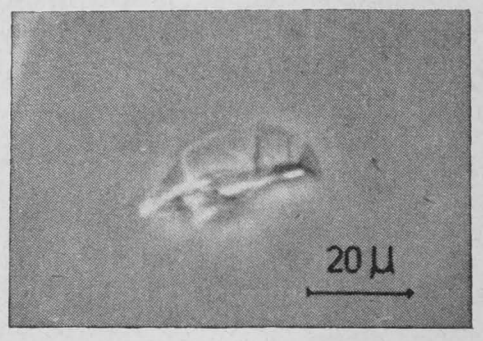

Fig. 3. Photomicrograph under phase contrast of a cuticle cell shown edge on, with an Allwörden sac produced by treatment with saturated chlorine water (see also ref. 10).

Fig. 3 the sac is fully developed on a detached cuticle cell, and covers an area which would be underneath the next overlapping cuticle cell in the intact fibre. The sac always occurs on only one side of the cuticle cell, for reasons which will be discussed in a subsequent paper. The occurrence of Allwörden sacs on partially and completely detached cuticle cells shows unequivocally that the epicuticle covers each cuticle cell separately and therefore cannot be regarded as continuous over the whole surface of the fibre.

Separated intact cuticle cells have been prepared from Merino and Lincoln wool, human hair, seal guard hair, kangaroo fur, platypus guard hair and platypus fur. Allwörden sacs of the type shown in Fig. 3 were produced in all cases, even though Allwörden sacs are not readily produced by chlorine water treatment of many types of intact fibres because of much overlapping and variation in shape of cuticle cells. The fact that the epicuticle membrane can always be observed by separation of the cuticle cell before treatment with chlorine water shows that it covers each cuticle cell separately on all keratin fibres.

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PUBLICATIONS:

The following publication has so far resulted from the work described in this thesis -

'Conformation of Epicuticle on Keratin Fibres',

Leeder, J. D. and Bradbury, J. H. (1968), Nature 218,694.

- a reprint is included.

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